# THE RESISTANCE OF RED CELLS IN VITRO

A Study of the Osmotic Properties, the Mechanical Resistance and the Storage Behaviour of Red Cells of Fetuses, Children and Adults

*By*

STIG SJÖLIN

UPPSALA 1954
ALMQVIST & WIKSELLS BOKTRYCKERI AB

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# Introduction

The *adult* red cells[1] have been studied extensively from different points of view, but comparatively little is known even now about the processes that are responsible for the normal red cell destruction. These processes may be divided into two different types, an extra-corpuscular one and an intra-corpuscular one. The shortened in vivo survival of the red cells in certain pathological conditions may be due to abnormalities in the one or the other of the processes mentioned. Of an extra-corpuscular nature are the specific hemolysins and agglutinins found in different acquired hemolytic anemias. Intra-corpuscular factors related to the structure of the red cell itself are of major importance for the decreased red cell survival appearing in diseases like congenital hemolytic anemia, in sickle cell anemia, and in thalassemia.

From in vitro experiments we know numerous ways in which the red cells can be destroyed or hemolysed. Whether or to what extent these processes are responsible for the normal red cell destruction in vivo is something about which we still have no definite ideas. The most important progress made in this field of research during the last few years is the introduction of reliable methods for determining the life time of red cells in vivo. Now it has become possible to compare the life time of different red cell populations with their in vitro behaviour.

In order to extend our knowledge concerning red cell destruction, more detailed in vivo and in vitro studies of normal and pathological red cells are needed. The more we learn about different types of red cells, of their composition, architecture and function, the closer we will reach an understanding of the real nature of the normal process of destruction.

The normal *fetal* red cell differs in many respects from the adult red cell. The shape and the hemoglobin differences are well known, but there are indications of other differences, which are not very well known or understood.

The primary purpose of this investigation has been to study the osmotic properties, the mechanical resistance and the storage behaviour of fetal red cells. There is reason to believe that fetal and adult red cells show differences in these properties, all of which are supposed to be correlated to the process

[1] In this paper the term "adult red cell" always means a red cell of an adult.

of red cell destruction in vivo. Another aim of the investigation has been to give, if possible, further information on the characteristic postnatal erythrocyte changes, the mechanisms of which are still in part obscure. Since there are no definite normal values for the osmotic and mechanical resistance of red cells from birth to puberty the study of these properties has been extended beyond the neonatal period.

In the first chapter a short survey of the properties of the adult and fetal red cells with special reference to characteristic differences will be given.

CHAPTER I

# A Short Survey of the Adult and Fetal Red Cell Properties, with Special Reference to their Characteristic Differences

## The Normal Adult Red Cell

### *Shape*

In one projection the adult red cell is circular, in the other it is biconcave. As a whole it has the shape of a symmetrical biconcave disc. This peculiar shape is the most suitable one for the respiratory function of the erythrocyte. Nobody has been able to explain how this shape is maintained. It is supposed to be dependent on the interior structure of the cell.

### *Dimensions*

The mean volume of the adult red cell is 87 $\mu^3$, its mean diameter 8.4 $\mu$, its mean thickness 2.4 $\mu$, and its mean surface area 163 $\mu^2$, all values referring to red cells in plasma.[1] The spherocyte index, i.e. the ratio mean thickness mean diameter, is about 0.27. In dry, stained films the mean cell diameter is 7.5 $\mu$ and the mean cell thickness 2.0 $\mu$, giving a spherocyte index of 0.26.

### *Metabolism and life time*

The erythrocytes are living cells, showing metabolic activity. They consume oxygen, the mature ones more slowly than the reticulocytes. The red cells also utilize glucose both by oxidation and fermentation, the end product in the last case being lactic acid. At present glycolysis is considered the most important, and maybe the sole process yielding energy. By different methods the life time of the normal red cell has been estimated to be about 120 days. The "age" is probably the most important mortality factor, but also random trauma may play a role.

### *Chemical composition*

The chemical composition of the red cell is fairly well known. It contains hemoglobin, other proteins, lipids, different electrolytes, glucose, urea, uric

[1] When no special reference is given in this paper, the normal values are taken from Albritton, Standard Values in Blood, 1952.

acid, creatin, creatinine, enzymes, and water. About 65 % of the total content is water and 35 % is solids. The hemoglobin constitutes 80 to 90 % of the solids.

*Interior*

Of the total interior volume, about 25 % is occupied by hemoglobin, the mean corpuscular concentration being about 34 g/100 ml. The interior water volume is about 70 %. As the most striking feature of the interior, PONDER (1948a) points out "the intimacy in which the hemoglobin molecules are situated with respect to one another". He also stresses that the ions "in the spaces between the hemoglobin molecules are subject to conditions very different from those obtaining in dilute solutions". According to PONDER, the system is on the threshold of gelation. Whether the interior of the red cell has any other structural component has been discussed. The protein stromatin has been supposed to form a structural network, but at present the question cannot be regarded as settled.

Another most remarkable feature of the red cell is its high potassium and low sodium content, in contrast to the low potassium and high sodium content of the extracellular fluid. Permeability studies with radioactive isotopes of potassium and sodium have definitely shown that both ions are readily permeable (SOLOMON, 1952). The concentration gradients therefore must be maintained by processes that for the present are interpreted as active ones, i.e. requiring energy expenditure. These processes are probably linked to the glucose metabolism. When the glucose has been exhausted, the transport mechanism fails.

*Membrane*

A lipo-protein-water membrane surrounds the interior of the cell. Electrophoretic studies have shown that the cell surface is dominated by acid groups. The detailed molecular arrangement of this membrane is not known. One of the most recent structural patterns is given by PARPART and BALLENTINE (1952). They describe tangentially oriented protein molecules in several layers and interspersed lipid molecules bound to the protein or to each other. The water content must be low. PARPART and BALLENTINE are of the opinion that the lipid parts of the membrane form pores. By rearrangement of the lipid molecules, these pores can be changed under special circumstances to continuous water channels going from the interior of the cell to the extracellular compartment.

The most recent theory of the ultrastructure of the red cell membrane, based mainly on electron microscope investigations, is presented by HILLIER and HOFFMAN (1953). They introduce a "plaque theory". The membrane

consists of plaques situated on the outside of a fibrous network joined together by lipids. The areas between the plaques are considered to be the pores. The plaque size is variable, on an average the diameter was estimated to be about 200 Å. The pore size varies with the plaque size, being for a plaque size of 200 Å about 35 Å.

The value of the total thickness of the membrane is rather uncertain, approximately 50–100 Å (Parpart and Ballentine 1952).

It is quite obvious that the membrane structure is closely related to the permeability properties of the red cell, but at present there is no complete picture of the functional mechanisms of the membrane. The membrane does not normally permit the passage of hemoglobin. The cations permeate slowly, the anions more rapidly, and water with great rapidity.

For more details concerning the normal adult red cell the reader is referred to Ponder (1948a), Parpart and Ballentine (1952), Gibson (1953), and Denstedt (1953).

## The Red Cells during Fetal Life

### *Erythropoietic periods*

In the young fetus most of the red cells are large primitive nucleated erythroblasts, which are produced in blood foci of the mesenchyme, especially in the yolk sac (Bloom and Bartelmez 1940). This mesoblastic period of hematopoiesis covers the first two to three months (Fig. 1). It is followed by the hepatic period, which is most active from the second to the sixth month. During this period the primitive cells are successively replaced by smaller ones, the mature nucleated normoblast, which are precursors of the ordinary erythrocytes. At the end of the second month about 50 % of the red cells are primitive erythroblasts, by the fourth month all primitive cells have disappeared. The third or the myeloid period begins in the fourth month (Knoll 1950).

### *Number and type of fetal red cells*

During the first months of fetal life the total concentration of red cells increases rapidly (Fig. 2). At the same time the nucleated cells and the reticulocytes decrease. In the middle of the third month there are about 8 % of nucleated red cells. After the fifth month less than 1 % are nucleated. From the late period of gestation only a few red cell and hemoglobin figures are available. The mean cell volume and the mean diameter decrease rapidly during the first three months, more slowly thereafter. The mean cell hemoglobin concentration seems to be fairly constant during fetal life, ranging between 31 and 34 g/100 ml of red cells. There is no in-

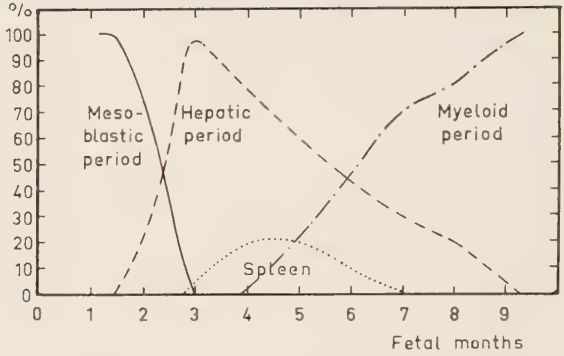


Fig. 1. The different stages of erythropoiesis and their relative magnitude during fetal life (after KNOLL 1950).

formation available about the shape of the fetal red cells during the hepatic and the myeloid periods. From the blood values of 30 fetuses, aged between 78 and 177 days, published by WINTROBE (1951), a spherocyte index of 0.24 in fresh red blood cells and 0.32 in stained ones can be calculated. Nothing is known about metabolism, life time, chemical composition, structure, and permeability properties of the erythrocytes during the early or the middle fetal life. (For literature concerning fetal red cells, see WINTROBE 1951).

## The Red Cells at Birth

### *Shape and dimensions*

In cord blood there are about 4.7 millions of red cells per $mm^3$. They are larger than the adult red cells, with a mean volume of 106 $\mu^3$ and a mean diameter of 8.0 $\mu$ in stained films. The mean cell hemoglobin concentration is about 33 g/100 ml (HORAN 1950). Their general shape is that of a biconcave disc. The above standard values give a spherocyte index of about 0.28.

### *Site of formation*

All of the placental red cells are fetal in origin, representative of the late fetal life.[1] There are, however, distinctly different kinds of fetal red cells at birth. Owing to the site of formation, there are at least two kinds, those formed in the bone marrow and those formed extramedullarily, mainly in the liver. Judging from the erythropoietic activity of the different sites (Fig. 1), the medullary type must be greatly in the majority. Whether these two types differ in any functional respect, we do not know at present.

[1] In the following the terms "fetal red cells" and "cord blood red cells" have been regarded as synonymous meaning the red cells of the cord blood of full-term infants.

*Hemoglobin*

With respect to the kind of hemoglobin, the fetal red cells are also of different nature. Of the red cells at birth, about 70 % contain the so-called fetal type of hemoglobin and about 30 % the normal adult type (Schulman and Smith 1953). The fetal hemoglobin has the same molecular weight as the adult hemoglobin (about 66,000), and the general shape of the molecules is also the same (Gutfreund 1949). In many other respects, however, these two types of hemoglobin show remarkable differences, all of which are supposed to be dependent on differences in the globin part of the hemoglobins (Lecks and Wolman 1950).

The fetal hemoglobin denaturates to alkali hematin much more slowly than the adult hemoglobin (Körber 1866). Their rates of spread in monolayers are different, the fetal type forms a stable layer more slowly. By using this spreading technique Brinkman and Jonxis (1935) found the isoelectric point of fetal hemoglobin to be 6.7 and that of adult hemoglobin 6.9. Electrophoretic studies have shown that the adult hemoglobin has a greater anodic mobility than the fetal hemoglobin (Beaven, Hoch and Holiday 1951). The two types are also distinguished by a different crystal form and by different solubility behaviour (Jope and O'Brien 1949). The oxygen dissociation curve of the fetal hemoglobin is shifted to the right of the adult curve, which highly favours the gas diffusion between mother and fetus (Smith 1951). Even their amino-acid composition (Porter and Sanger 1948) and their immunologic properties (Darrow *et al.* 1940) are different. Nothing is known concerning the possible differences in the site of formation of fetal and adult hemoglobin.

With the exception of the shift of the oxygen dissociation curve, the physiological significance of the special fetal hemoglobin is not known. In this connection, however, it is of interest to mention that other atypical hemoglobins have been found in some diseases that are characterized by red cells with a shorter life time than normal, dependent on intrinsic defects of the red cells. Such diseases are sickle cell anemia and thalassemia, and also some cases of hereditary spherocytosis (Singer, Chernoff and Singer 1951).

Whether there are any other remarkable differences in chemical composition or in structure between the late fetal red cells and the adult red cells, we do not know. In the red cells of sixteen children aged 5–9 years, Erickson *et al.* (1937) found an increased potassium concentration, when calculated in terms of meq. per liter cell water, and it does not seem unlikely that the red cells at birth also differ from the adult cells with regard to their mineral content.

*Metabolism*

The metabolism of fetal red cells has not roused much interest. The only investigation on this subject is that of FÅHRÆUS (1953), who showed that the activity of the acid phosphatases of laked red cells from newborn infants (cord blood) had a lower temperature optimum than laked red cells from adults.

## The Red Cells of the Newborn

*Changes in number and type*

During the first two hours after birth, there is a temporary rise in the red cell count, in the hemoglobin values (Fig. 2), and in the number of reticulocytes. The mean cell volume and the mean cell diameter both show a slight rise (WEGELIUS 1948, WEICKER *et al.* 1953).

After the first three to four days of life and until the second month the number of red cells per $mm^3$ steadily declines. The hemoglobin percentage falls more rapidly than the number of red cells (Fig. 2). The number of nucleated red cells and of reticulocytes, which is rather high at birth, falls rapidly after the first or the second day, and at the end of the first week of life there are no signs of a hyperactive red cell production (FAXÉN 1937; GAIRDNER, MARKS and ROSCOE 1952). At the same time the hemopoietic tissue of the liver has disappeared in full-term infants (LANGLEY 1951). The mean cell volume and the mean diameter decrease steadily. The mean cell hemoglobin concentration seems to be practically constant during this period (HORAN 1950). The percentage of fetal hemoglobin starts to diminish after birth, and at the age of 6–7 months practically all fetal hemoglobin has disappeared (BRINKMAN and JONXIS 1935).

*"The physiological anemia"*

The real nature of this striking decrease in hemoglobin and red cells during the first months of life has been much discussed during the last decades. For details, the reader is referred to the papers of FINDLAY, HIGGINS and STANIER (1947), MOLLISON (1948), KÜNZER (1951), MOLLISON (1951), and SMITH (1951).

For a long time it was taken for granted that the so-called physiological anemia and the hyperbilirubinemia of the newborn were the results of a rapid and excessive red cell destruction starting immediately after birth. At present there is, however, no convincing evidence of such an *excessive* hemolysis.

By the differential agglutination method of ASHBY (1919), MOLLISON in 1948 made a first attempt to measure the life time of the red cells of newborn infants. He concluded that the majority of the cells had the same life time as the

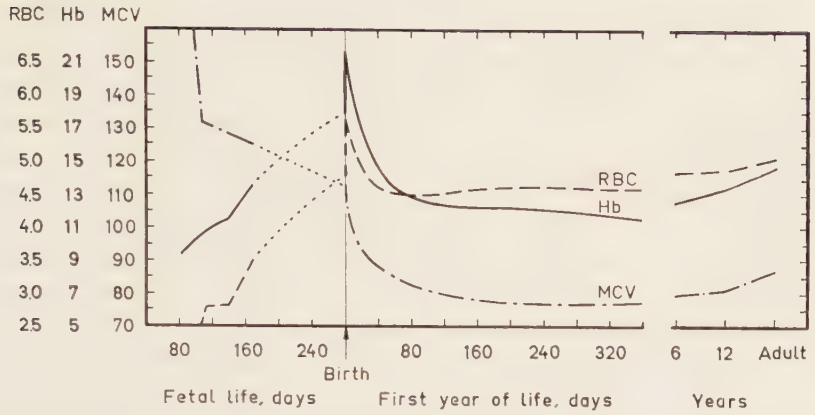


Fig. 2. Variations in red blood cell count (RBC, millions per $mm^3$), hemoglobin concentration (Hb, g per 100 ml red cells), and meán red cell volume (MCV, $\mu^3$) during different ages. (The values obtained from ALBRITTON 1952, and WEGELIUS 1948).

adult red cells, but he also drew the conclusion that there was a minority of cells with a shorter life time. After improving the procedure by transfusing a control population of adult red cells together with the cord blood cells and by using only venous blood for the counting, MOLLISON continued his work. In 1951 new experiments were published. They were interpreted in the same way: "Some, at least, of the red cells produced during late fetal life survive for a shorter time than adult red cells." This statement seems convincing.

The fetal and adult hemoglobins in premature infants have been studied by SCHULMAN and SMITH (1953). They too, from their point of view, were able to conclude that "the initial red blood-cell population present in the premature infant has a decreased survival time".

Today it seems very probable that at least some of the fetal red cells have a shorter life time than the adult ones. This, however, cannot be the only explanation of the physiological anemia. Another, and possibly the main factor responsible for the decreasing post-natal erythrocyte count is the suppression of the erythropoietic activity of the bone marrow, together with the post-natal falling off of the extramedullary hematopoiesis (VAHLQUIST 1941; LANGLEY 1951; GAIRDNER, MARKS and ROSCOE 1952). The physiological anemia may also partly be explained by a dilution of the blood during the first month of life (STAHLMAN, KARLBERG and LIND 1951). As to the physiological icterus of the newborn, most studies positively support the view that the hepatic immaturity is the main cause (FINDLAY *et al.* 1947).

CHAPTER II

# The Osmotic Resistance of the Red Cells of Fetuses, Children and Adults

Special credit should be given to JACOBS, PARPART, PONDER, and WILBRANDT for their contributions to our knowledge of the osmotic properties of the adult red cell. PONDER's "Hemolysis and related phenomena" (1948a) and the series of papers by JACOBS *et al.* (1930–52) contain much information about the osmotic properties of the red cells.

## The Process of Hemolysis

As an introduction to this chapter a short, general description of the hemolysis in hypotonic NaCl solution is appropriate.

In a hypotonic NaCl solution the red cell takes up water in order to maintain iso-osmotic conditions. Owing to the apparent electrolyte semipermeability of the red cell membrane no electrolytes are lost, and the cell swells until osmotic equilibrium between the cell and the hypotonic media is regained. The degree of swelling depends on the degree of hypotonicity. If the red cell were an ideal osmometer, the swelling would follow the "van't Hoff-Mariotte law", which for the present purpose could be written

$$C\,V = C_0 V_0, \tag{1}$$

where $C_0$ is the initial osmolar concentration, $V_0$ the initial "osmotically effective volume" of the cell, and $C$ and $V$ are the corresponding new concentration and volume (the solution is assumed to behave ideally in the sense that activities can be substituted by concentrations).[1] In practice the red cells sometimes are nearly perfect osmometers, sometimes quite imperfect. GUEST (1948) has been able to show that normal human red cells from heparinized blood follow the equation (1) in NaCl solutions in concentrations of 0.900 % to 0.425 % (153.9–72.7 mM). The same was found to be the case with red cells from children and newborn infants. In concentrations below 0.425 % the osmometer function is limited by the hemolysis. Red cells from

[1] See also p. 19.

oxalated blood and red sickle cells, on the contrary, do not follow the equation (1), i.e. they are imperfect osmometers (PONDER 1948a; GUEST 1948). During the swelling the red cell changes shape. Crenations appear and the biconcave disc becomes more and more spherical. During the first stage of swelling the cell diameter decreases continuously, the surface area being practically constant. In the second stage "some structure seems to give way suddenly" (PONDER 1948a, p. 82), the cell becomes a sphere and the area decreases momentarily. The third stage of swelling is characterized by a continuous increase in diameter and surface area of the sphere. When the area of the sphere has become approximately the same as that of the initial disc, the so-called critical volume is reached. In the very moment of reaching this volume the membrane is stretched and the cell starts to lose its hemoglobin, i.e. the cell hemolyses.

The osmotic properties of the red cells can be studied in different ways. Determination of the red cell fragility or resistance in different degrees of hypotonicity is the method most commonly used. This test, however, gives information only of the *end state* of the process of hemolysis. For a more complete understanding of the process it is of importance to know also the *rate* at which this end state is reached, or in other words, the rate of entrance of water into the cell (JACOBS 1932a). Both aspects of the process of hemolysis will be dealt with here, firstly the resistance and, secondly, in Chapter IV, the kinetics of hemolysis in hypotonic media.

## Earlier Investigations

The osmotic resistance of the red cells from infants and children has been the subject of numerous investigations. There is, however, a remarkable lack of conformity in the results.

In *cord blood*, GOLDBLOM and GOTTLIEB (1929) found a markedly lowered resistance of the red cells in comparison with the adult red cells. FINDLAY (1945) could show but a slightly diminished resistance. GENTILI and FASCIONE (1948) observed a few erythrocytes of minimal resistance in the fetal blood, while the number of those of maximal and average resistance were increased. CRAWFORD, CUTBUSH and MOLLISON (1953) found the value of 50% hemolysis to be the same in cord blood and in adult blood, while 5% hemolysis was often seen at a higher concentration in cord blood than in adult blood. Other authors have not observed any differences (MITCHELL 1928).

An increased span of hemolysis at birth, i.e. an increased range between the beginning and the complete hemolysis, has been demonstrated by SIMMEL and SIMMEL-RAPP (1924) and by WAUGH, MERCHANT and MAUGHAN (1939).

The earlier investigations on the resistance of the red cells during *infancy and childhood* also reveal divergency of opinion. SIMMEL and SIMMEL-RAPP (1924) found a high number of resistant cells during the first month of life thereafter the resistance decreased, but as a whole an increased resistance was noticed until puberty. WHITBY and HYNES (1935) studied 10 infants during the first 24 hours after birth. They stated that some cells are less resistant and others more resistant than adult red cells. In a study of 167 infants during the first 9 days of life, WAUGH, MERCHANT and MAUGHAN (1939) found a greater resistance during the first four days. Thereafter the values tended to return towards normal. After investigating 50 children aged between 2 months and 12 years, CORMICK (1942) stated that the osmotic resistance of the red cells during this period was comparable with the resistance of adult red cells. In four infants, investigated during the first week of life, he found a slightly decreased resistance in the lower concentrations. At the age of 11 days the osmotic resistance had reached the values found in the older infants. In a group of 85 children aged from one day to 12 years, FINDLAY (1945) observed a slight increase of resistance. An increased resistance compared with normal adults was found by CRAWFORD, CUTBUSH and MOLLISON (1953) in infants aged 2–5 days.

None of these investigators has tried to interpret or discuss the results with regard to their significance.

No investigations have been published concerning the osmotic resistance of red cells from the early or middle fetal life.

There is obviously good reason for agreeing with SMITH (1951), who states that "data as to the relative fragility of erythrocytes during the newborn period are rather unsatisfactory".

The divergencies of opinion are not difficult to explain. Only a few authors have observed or taken into account the rapid change in osmotic resistance immediately after birth and they make no distinction between cord blood and blood of the first days of life.

There is also a common tendency to look on all the cells as a whole, describing their resistance as either increased, normal, or decreased in comparison with the adult red cells. A more detailed analysis might have revealed that the total red cell population was built up of smaller populations with different osmotic properties.

The diversity of opinions may also originate from the rather unsatisfactory methods often used. Many factors that strongly influence the osmotic resistance of the red cells were not known to previous investigators. Few authors have used the same method, and their results have thus not been quite comparable.

The most important methodological factors will be discussed in brief.

## Methodological Problems

In order to characterize the whole red cell population the method must give quantitative figures by which a *percentage hemolysis curve* can be plotted. The determination of the beginning and the complete hemolysis concentrations only, as a rule done in the clinic, is unsatisfactory, since both these points are very difficult, not to say impossible, to determine exactly. A percentage hemolysis curve is most conveniently made by determining the concentration of the hemoglobin lost to the supernatant fluid in a series of hypotonic saline solutions after a given time. Counting the unhemolysed cells is a less sensitive and a more time-consuming procedure.

Heparin is preferred as an *anticoagulant*. In oxalate the red cells do not behave as osmometers, and with citrate slight hemolysis is risked.

The relative *amount of plasma and saline* must also be considered. If there is much plasma in relation to saline, the salt-containing plasma will influence the final saline concentration. Therefore, either the same proportion of cells to plasma or very small amounts of cells and plasma in relation to saline have to be used.

The *unit of amount* has frequently been a "drop" of blood. In quantitative work such an inexact measure must be avoided. Instead, one has to use a constant amount of cells, washed or unwashed, in a constant amount of saline.

There are also a few still more important factors to consider. Red cells from arterial blood are known to be more resistant than those from venous blood. It has been shown that this higher resistance depends on the lower carbon dioxide and the consequently higher pH in arterial blood. Some authors have tried to overcome this problem by oxygenating the blood in air.

A better way to obtain accurate results is to use very small quantities of blood in relation to saline and well-buffered saline solutions (Jacobs and Parpart 1931). A dilute hemolytic system is very sensitive to *changes in pH*. An increase of pH gives an increase in osmotic resistance. A change in pH of 0.1 between pH 6.0 and 8.5 corresponds to a change in saline concentration of about 0.010 % (1.7 mM).

The degree of *oxygenation* in itself plays only a minor role. Completely oxygenated red cells are more resistant than the completely reduced ones. Laboratory saline solutions, however, are in equilibrium with the room air, and in such solutions hemoglobin will be almost completely oxygenated. When the pH is constant, special precautions are not needed with respect to oxygenation.

The osmotic resistance is also strongly influenced by the *temperature*. The lower the temperature, the lower the resistance. In Jacobs' and Parpart's experiments (1931) a change in temperature of 10° C in the region from 0° to + 40° C corresponded to a concentration change ranging from 0.020–0.044 % NaCl (3.5 mM–7.5 mM).

The described connection between the osmotic resistance, the pH and the temperature is valid for all degrees of hemolysis. Accordingly, the percentage hemolysis curve shows a practically parallel shift, when pH or temperature are altered (Parpart *et al.* 1947).

A critical evaluation of the methods used by previous investigators shows that only those of Waugh, Merchant and Maughan (1939) and of Crawford, Cutbush and Mollison (1953) are incontestable. Against the methods of the other authors more or less serious objections can be raised.

If all the discussed aspects of the osmotic resistance test are taken into account, it is at present possible to get reliable and detailed information of the erythrocyte behaviour in hypotonic saline solutions.

## Methods

1. *The blood samples* were collected in Erlenmeyer flasks (Pyrex) containing heparin as an anticoagulant (0.05 ml 1 % heparin per 10 ml blood). It was not possible to take the blood samples at a time fixed in relation to the meals.

In adults and children venous blood was obtained from the cubital or femoral veins. In infants, blood from femoral vein or "capillary" blood from a heel puncture was used. The heel puncture with a sharp stilette gave a rapid dropping of blood after a thorough warming of the foot. At birth, blood was obtained from the placental part of the umbilical cord immediately after the delivery. In fetuses, blood was collected from the fetal part of the cord immediately after surgical abortion.

2. *The hemoglobin of whole blood* (Hb) was measured as the oxyhemoglobin concentration in light of wave length 550 600 with a photoelectric hemoglobinometer, Haemotest (Testalaboratorium, Vedbæk, Danmark). The error was found to be ± 3.6 % when calculated from 100 arbitrarily chosen duplicate determinations.[1]

3. *The number of red cells* (RBC) was counted according to the usual routine clinical method. According to Berkson (1944) the error is ± 16 %. Biggs and Mac Millan (1948) found a variation of the error of the red cell count between ±2 and ± 9.5 % depending on the training of the workers, who made the counts. With a scrupulous standardized technic an error of about ± 1 $^1/_2$ % was obtained by Enghoff (1937).

4. *The mean red cell diameter* (MCD) was measured in 1000 × magnified photographs of dried blood films. According to Larsen (1948) the error of MCD can be estimated to be about 1.5 %.

5. *The packed red cell volume* (PCV) was determined in the high speed hematocrit centrifuge of Enghoff (1937) (3 minutes at 9600 r.p.m. with a mean radius of 10 cm). The error was found to be ± 1.4 % when calculated from 100 arbitrarily chosen duplicate determinations.

6. *The mean red cell volume* (MCV) was calculated from the formula

$$\mathrm{MCV} = \frac{\mathrm{PCV} \times 10}{\text{red cell count, millions}}\ \mu^3$$

7. *The mean red cell thickness* (MCT) was calculated from the formula (regarding the cell as a short cylinder)

$$\mathrm{MCT} = \frac{\mathrm{MCV}}{\left(\frac{\mathrm{MCD}}{2}\right)^2 \cdot \pi}\ \mu$$

8. *The spherocyte index* is a measure of the degree of spherocytocysis and is expressed as the ratio $\frac{\mathrm{MCT}}{\mathrm{MCD}}$.

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[1] According to $\sigma = \pm \sqrt{\frac{\Sigma d^2}{2n}}$ (*cf.* Enghoff 1937).

9. *The reticulocytes* were stained with brilliant cresyl blue in a small test tube for 30 minutes. A thin film of this mixture was prepared on a slide. The number of reticulocytes per 1000 red cells was recorded.

10. *The fetal hemoglobin* concentration was measured by an alkali denaturation technic. The method of SINGER, CHERNOFF and SINGER (1951) was used. The error of a single reading determined from twenty identical red cell suspensions was $\pm$ 4.0 % ( $+ \sigma$).

11. *The pH* of the buffer was determined with a glass electrode in a potentiometer (type PHM3a, Radiometer, Copenhagen).

12. In *osmotic resistance tests* it would be most correct to use diluted plasma as the hypotonic medium. Because it was difficult to obtain a sufficient amount of plasma, buffered NaCl solutions were used instead.

Using a thermoelectric vapour tension method, LIFSON (1944) found the total osmotic activity of plasma to be 155.5 meq. per kilo. A NaCl solution of the same concentration is isotonic with plasma. Following PONDER (1948 a) a slightly hypertonic NaCl solution, 171.1 mM, has been regarded as iso-osmotic with plasma in this paper.

The osmotic activity of the hypotonic NaCl solutions used can be assumed to be directly proportional to their NaCl concentrations. The coefficients of osmotic activity are practically the same for all solutions used; for the highest concentration (171.1 mM) it was calculated to be 0.90, and for the lowest one to be 0.94. Data of activity coefficients ($f$) were obtained from CONWAY (1952) and the osmotic coefficients ($g$) were calculated by the formula $-\ln f = 3(1-g)$ (BJERRUM 1916, 1923; quoted from HÖBER 1945).

The best expression of concentration is that which permits a direct comparison between different kinds of solutions, i.e. number of moles per volume. Such expressions are for instance molarity (M) or millimolarity (mM). In this paper, however, concentrations will be expressed as g per 100 ml (= %) on account of the common clinical use. Important figures of concentrations, however, will be transformed to mM. For NaCl the following equation expresses the transformation

$$\text{mM} = \frac{\%\ \text{NaCl} \times 10 \times 1000}{58.45}$$

The *osmotic resistance was determined by the method of* PONDER (1948 b) with some modifications, of which the following are the most important. For practical reasons it was not possible to determine the hemolysis after 5 hours; instead, the time of exposure was changed to 20–22 hours. In consequence the temperature had to be changed from + 25° C to + 4° C and, as the same buffer was used, the pH changed from 7.0 to 6.8. In order to save time whole blood was used instead of washed red cells.

A series of *hypotonic NaCl solutions* was prepared by diluting a 5 % (855.4 mM) NaCl solution with distilled water. The series usually started with a 0.68 % NaCl solution and descended successively by a difference of 0.04 % to a lowest value of 0 %. The NaCl solutions were stored in stoppered Pyrex bottles at room temperature (20° C). They were renewed at least every fortnight.

A *buffered NaCl solution* was made by mixing 25 ml of a sodium phosphate buffer and 75 ml of a NaCl solution with a concentration of 1.2 g/100 ml (205.3 mM). The sodium phosphate buffer was made by mixing 18 ml of M/15 $Na_2HPO_4$ (66.6 mM)

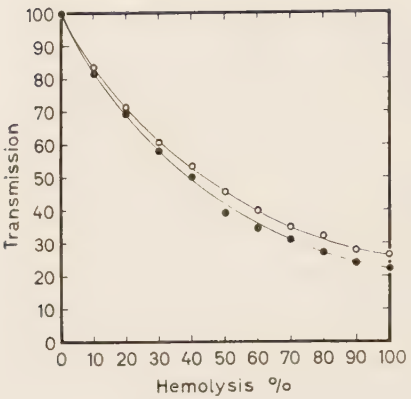



Fig. 3. Relation between hemolysis and transmission. Calibration curves from two different blood samples.

and 7 ml of M/15 $NaH_2PO_4$ (66.6 mM). The total tonicity of this buffer equals an 1% NaCl (171.1 mM) solution. At + 4° C the pH of the buffer is 6.8. The buffer solutions were stored in stoppered Pyrex bottles at + 4° C and were renewed at least every fortnight.

A *red cell suspension* was prepared of whole blood (for special purposes, red cells washed twice in 1% NaCl were used) and the buffered NaCl solution, so that the volume of red cells became 0.025 ml per ml solution. The amount of whole blood containing the desired amount of red cells was calculated from the packed red cell volume.

A *hemolytic system* was made by mixing 1 ml of this red cell suspension and 4 ml of the different hypotonic NaCl solutions. The concentration of red cells thus became 1/200. The *final* saline concentration of the hemolytic system is easy to calculate (e.g., 1 ml 1% red cell suspension + 4 ml 0.68% NaCl gives a final concentration of $(1 \times 1 + 4 \times 0.68) : 5 = 0.744\%$). The hemolytic series started as a rule at 0.744% (127.3 mM) and descended by 0.032% (5.47 mM) to a lowest value of 0.200% (34.2 mM).

The *percentage hemolysis curve*. The hemolytic series was prepared at room temperature and thereafter immediately stored at + 4° C for 20–22 hours. The red cells were then centrifuged off (room temperature, 10 minutes, 3000 r.p.m.). Four ml of the supernatant fluid were mixed with 0.01 ml $NH_4OH$ and exposed to CO for half a minute. The CO-Hb concentration of the supernatant fluid was determined in a photoelectric colorimeter (selenium barrier photocell) (Teorell 1952) using parallel blue light (Jena filter BG 18 with maximal transmittance at 508 mμ). A cuvette of 1.00 cm length, at a distance of 2 cm from the photocell, was used. The colorimeter operated a short period galvanometer and a Beckman Photopen-Recorder was used as reading instrument. The CO-Hb concentration was expressed as percentage of transmission. The percentage of hemolysis was obtained by means of a *calibration curve*, which was made from the transmission values of samples of known degree of hemolysis. A series of tubes of 100, 90, 80 ... and 0 % hemolysis was prepared by freezing (— 18° C) and thawing 2.0 ml, 1.8 ml, 1.6 ml etc. of the red cell suspension and then mixing these hemolysed samples with 8.0 ml, 8.2 ml, 8.4 ml etc. of 1% NaCl. In this way calibration curves were made for different bloods. All these curves had the same slope, but depending on small variations in hemoglobin concentration they were situated at different levels (Fig. 3). In most experiments only the transmission of 0% and 100% hemolysis was determined. The general slope of the calibration curves

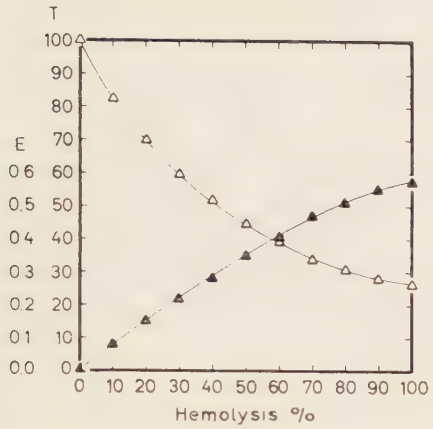



Fig. 4. Shapes of the calibration curves. Transmission (△); extinction (▲).

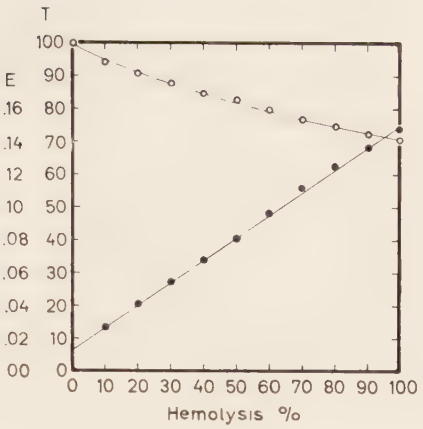



Fig. 5. Effect of dilution on the calibration curves. Transmission (○); extinction (●).

being known, it was possible to interpolate the whole curve from these two observed transmission values.

The experimentally obtained transmission curves were not linear. Due to the high concentration used not even the corresponding extinction curves were linear. They did not obey the Lambert-Beer's law unless the fluid was diluted four times (Figs. 4 and 5). The definite percentage hemolysis curve, however, has been shown to be the same, whether the calibration curve from the diluted or from the undiluted solution was used. In this situation the calibration curves based on the direct transmission readings were used throughout, because this procedure was the most time-saving one.

The *error* of a single reading determined from twenty identical blood suspensions was for 46 % hemolysis = 3.5 %, for 78 % = 1.9 %, and for 96 % = 2.5 % ($\pm\sigma$).

## Statistical Treatment of Percentage Hemolysis Curves

The statistical treatment of the percentage hemolysis curves can be performed in different ways. In this investigation slightly different methods have been used for different types of curves. The most complete analysis has been given to the adult and the cord blood curves.

In *adults* the procedure was based on the fact that the osmotic resistance of red cells is normally distributed to the NaCl concentration. This means that the percentage hemolysis curve has a symmetrical sigmoid shape (Fig. 6). The probit curve of such a sigmoid curve is a straight line (Gaddum 1933) (Fig. 10 b). The mean values of the percentage hemolysis in the different NaCl concentrations were calculated from the observed values and transferred to the corresponding probit values (Finney 1947). By the method of least squares the straight line was estimated and the "standard error of estimate" was

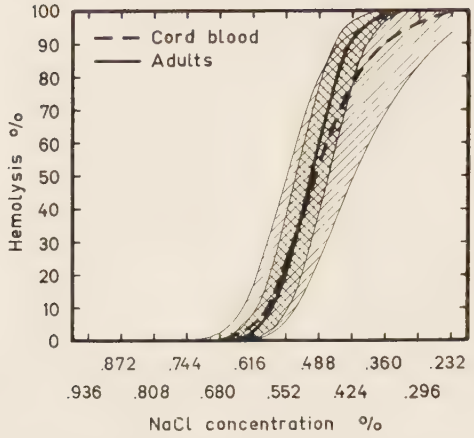



Fig. 6. Degree of hemolysis of the red cells of adults and newborn infants (cord blood) at different NaCl concentrations. The mean percentage hemolysis curves are given (heavy lines). The shadowed areas comprise 95.44 % (= ± 2 SD) of the material.

calculated.[1] The mean probit line and the lines representing twice the "standard error of estimate" was then transferred to percentage hemolysis curves (Fig. 6). In this way a surface, comprising 95.44 % of the total normal material for adults, was obtained. This surface is of value when individual curves of different kinds have to be compared with the normal adult curve.

For a complete statistical comparison of different percentage hemolysis curves some of their parameters have to be calculated. From the sigmoid percentage hemolysis curve one can read the saline concentration that causes 10, 20, 30, or every other percentage of hemolysis. In conformity with the generally accepted terms $LD_{50}$ and $ED_{50}$ these saline concentrations will be represented in this paper by $H_{10}$, $H_{20}$, $H_{30}$, etc. (H standing for hemolysis). From the mean percentage hemolysis curve the mean values of $H_{10}$, $H_{50}$, and $H_{90}$ were obtained. The mean value of $H_{50}$, found from the mean percentage hemolysis curve (0.504 % NaCl), was compared and found to agree very well with the mean value of $H_{50}$ calculated from the individual curves (0.517 % NaCl). The deviation of $H_{50}$ obtained from the curves of the "standard error of estimate" was also found to agree very well with the standard deviation of $H_{50}$ calculated from the individual means of $H_{50}$ by common methods ($\pm$ 0.015 % compared with $\pm$ 0.025 % NaCl). In this situation the graphically obtained values of the mean percentage hemolysis curve were chosen, since this curve was based on a mathematical treatment of all observations.

As a measure of the *span of hemolysis* most authors use the lower and the upper extreme (range) of the curve. These values, however, are impossible

[1] The weight for different percentage hemolysis ranges is, of course, varying (see Gaddum, 1933, and Finney 1947), but in the present calculations these differences in weights were neglected, because they could not be estimated in each case (cord blood cells). A comparison between lines for adult red cells, calculated with and without the weights, justified the simplified calculation used.

to estimate exactly and indeed they are connected to infinite high and low concentrations, i.e. the 0 % and 100 % lines are asymptotes to the curve. For this reason this way of representing a hemolysis curve is unsatisfactory and should be abandoned. Instead, any other statistical expression of dispersion, e.g. the standard deviation, the quartile deviation, the difference between the highest and the lowest deciles, can be applied. In the following, the difference between the highest and the lowest deciles ($D_9 = H_{90}$ and $D_1 = H_{10}$) will be used. This difference will be called the *extreme decile difference.*

From the median ($M = H_{50}$) and the two deciles the possible *skewness* of the percentage hemolysis curve can be calculated in analogy with the formula given by YULE and KENDALL (1945) and valid for the quartiles

$$\frac{(D_9 - M) - (M - D_1)}{(D_9 - M) + (M - D_1)}$$

With the terminology used here this formula can be written as

$$\frac{(H_{90} - H_{50}) - (H_{50} - H_{10})}{(H_{90} - H_{50}) + (H_{50} - H_{10})}$$

The distribution is said to be positively skew, when the curve is more extended to the right than to the left and vice versa.[1] This is in accordance with the common graphical picture of such curves.

*In this way the most important characteristics of a single percentage hemolysis curve or of a collection of such curves have been obtained. The values needed are those of* $H_{10}$, $H_{50}$ *and* $H_{90}$.

The osmotic resistance of red cells from the *cord blood* are, as will be shown later, not normally distributed to the NaCl concentration. A procedure of statistical treatment that in some way differs from the treatment of the adult curves has therefore been adopted. The mean values of the percentage hemolysis in the different NaCl concentrations were calculated from the observations of all cases and transferred to the corresponding probit values. The line best fitting these probit values was drawn by hand. Each experimental observation was then transferred to probit values. The distance between each experimental probit value and the constructed probit line was estimated and the sum of the square differences was used for calculating the "standard error of estimate". The two curves of twice the "standard error of estimate" were drawn. These three probit curves were transferred to percentage hemolysis curves (Fig. 6). Thus the mean percentage hemolysis curve and the surface comprising 95.44 % of the material were obtained. From these

[1] Note, however, that the $x$-axis is decreasing to the right.

curves the mean value of $H_{10}$, $H_{50}$ and $H_{90}$ and their deviations were obtained. The extreme decile difference and the skewness were calculated as before.

In *fetuses, infants and children* a simpler procedure was used. Only the main characteristics necessary for the description of the results were calculated. From the individual percentage hemolysis curves the mean values of $H_{50}$ and their standard deviations were obtained. The mean values of $H_{10}$ and $H_{90}$ were obtained from the mean percentage hemolysis curves. The extreme decile difference and the skewness were then calculated.

*Frequency distribution* curves were obtained by differentiation of the smoothed percentage hemolysis curves. The differentiation was carried out graphically by means of a "tangent meter".

For *comparison of two means* the $t$ test was applied. The significance of a difference of two means is given as the probability ($P$) of the means not to be separated. For example, if $P$ is found to be $< 0.01$, this signifies that there is less than one chance in a hundred for the means to belong to the same distribution. In the following, the term "significant" will sometimes be used. Those cases refer to a $P \leq 0.01$.

## Material

The *adult* material consisted of healthy nurses, laboratory assistants, doctors, and medical students.

The *infants and children* investigated were treated at the Children's Clinic or at a home for children (Gillbergska barnhemmet) in Uppsala. Most of these infants and children were healthy, only a few suffered from epilepsy or other neurological diseases. None showed any signs of blood diseases or of infectious diseases.

The cord blood samples and the blood samples from the *newborn infants* were obtained from the Gynecology Clinic, Uppsala. All these infants and their mothers were healthy.

The *fetus* material was collected from surgical abortions carried out at the Gynecology Clinic, Uppsala. Specimens from abdominal Caesarean sections only could be used. None of the women operated suffered from any somatic disease.

## Results

The results of the osmotic resistance investigations during different ages are shown in Table 1 and Figures 6–12. Some points of special interest will be reported below.

### *Adults*

The osmotic resistance of 47 adults was determined by the present method in order to obtain a normal material for comparison. The mean percentage

TABLE 1

The osmotic resistance of the red cells of fetuses, children and adults.

| Age group | No. of cases | $H_{10}$ % NaCl Mean | $H_{50}$ % NaCl Mean | $H_{50}$ % NaCl SD | $H_{50}$ mM Mean | $H_{90}$ % NaCl Mean | Extreme decile difference | Skewness |
|---|---|---|---|---|---|---|---|---|
| Fetuses . . . | 13 | 0.700 | 0.584 | ±0.043 | 100.0 | 0.424 | 0.276 | +0.16 |
| Cord blood . . | 31 | 0.588 | 0.496 | ±0.032 | 84.9 | 0.366 | 0.222 | +0.17 |
| 12–48 hours . | 20 | 0.552 | 0.446 | ±0.033 | 76.3 | 0.322 | 0.230 | +0.08 |
| 6–8 days . . | 11 | 0.568 | 0.468 | ±0.051 | 80.1 | 0.354 | 0.214 | +0.07 |
| 15–45 days . . | 11 | 0.550 | 0.471 | ±0.026 | 80.6 | 0.382 | 0.168 | +0.06 |
| 46–75 days . . | 12 | 0.548 | 0.466 | ±0.023 | 79.7 | 0.376 | 0.172 | +0.05 |
| 76–105 days . | 15 | 0.548 | 0.469 | ±0.035 | 80.2 | 0.386 | 0.162 | −0.04 |
| 4–6 months . | 10 | 0.548 | 0.469 | ±0.034 | 80.2 | 0.386 | 0.162 | −0.04 |
| 10–12 months . | 11 | 0.526 | 0.438 | ±0.030 | 74.9 | 0.352 | 0.174 | −0.01 |
| 2–5 years . . | 10 | 0.556 | 0.477 | ±0.034 | 81.6 | 0.396 | 0.160 | +0.01 |
| 7–11 years . | 16 | 0.532 | 0.465 | ±0.020 | 79.6 | 0.398 | 0.134 | 0.00 |
| Adults . . . . | 47 | 0.572 | 0.504 | ±0.015 | 86.2 | 0.438 | 0.134 | −0.01 |

*t* tests on the differences of $H_{50}$

| Compared age groups | Difference % NaCl | Probability of identity |
|---|---|---|
| Fetuses *vs.* cord blood . . . . . | 0.088 | $P < 0.001$ |
| Cord blood *vs.* 12–48 hours . . . . . | 0.050 | $P < 0.001$ |
| Cord blood *vs.* 6–8 days . . . . . | 0.028 | $P < 0.10$ |
| Cord blood *vs.* 15 days–6 months . . . . . | 0.027 | $P < 0.001$ |
| Cord blood *vs.* 10–12 months . . . . . | 0.058 | $P < 0.001$ |
| Cord blood *vs.* 2–11 years . . . . . | 0.026 | $P < 0.001$ |
| Cord blood *vs.* Adults . . . . . | 0.008 | $0.10 < P < 0.50$ |
| 6–8 days *vs.* 12–48 hours . . . . . | 0.022 | $0.10 < P < 0.50$ |
| 15 days–6 months *vs.* 12–48 hours . . . . . | 0.023 | $P < 0.01$ |
| 12–48 hours *vs.* 10–12 months . . . . . | 0.008 | $0.10 < P < 0.50$ |
| 6–8 days *vs.* 15 days–6 months . . . . . | 0.002 | – – – |
| 15 days–6 months *vs.* 10–12 months . . . . . | 0.031 | $P < 0.001$ |
| 15 days–6 months *vs.* 2–11 years . . . . . | 0.001 | – – – |
| 2–11 years *vs.* 10–12 months . . . . . | 0.032 | $P < 0.001$ |
| 2–5 years *vs.* 7–11 years . . . . . | 0.012 | $0.10 < P < 0.50$ |
| Adults *vs.* 2–11 years . . . . . | 0.034 | $P < 0.001$ |

hemolysis curve had a sigmoid shape (Fig. 7) and the frequency distribution of the resistance was practically normal (Fig. 8). The same was the case in the majority of the individual curves; in a few only the distribution was slightly skew in either direction.

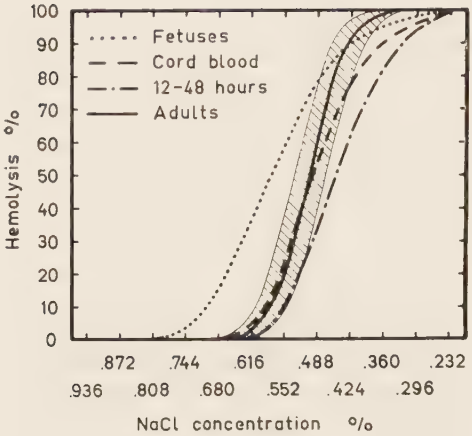



Fig. 7a. Degree of hemolysis of the red cells of fetuses, newborn infants (cord blood), infants aged 12–48 hours, and adults at different NaCl concentrations. The mean percentage hemolysis curves are given. The shadowed area comprises 95.44 % of the adult material.

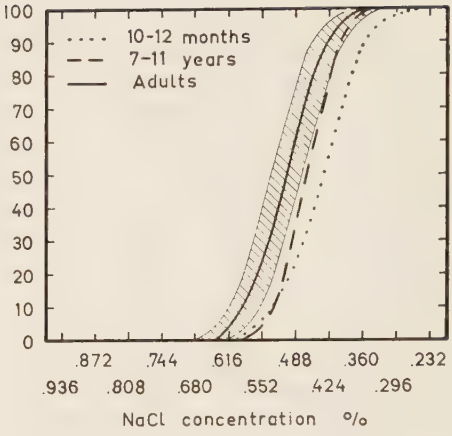



Fig. 7b. Degree of hemolysis of the red cells of infants aged 10–12 months, children aged 7–11 years, and adults at different NaCl concentrations. The shadowed area comprises 95.44 % of the adult material.

A detailed comparison of the normal values obtained by the present author and other investigators is not possible, because the differences between the methods of different authors result in differences in osmotic resistance that cannot be expressed in exact figures of % NaCl. Only a rough comparison can be made.

Ponder (1948 b), using washed red cells by a pH of 7.0 and a temperature of 25° C, obtained symmetrically distributed resistance curves and a mean value of 0.448 % for $H_{50}$. Remembering that 0.01 pH $\simeq$ 0.010 % NaCl and 10° C $\simeq$ 0.020 % NaCl, we have to add 0.060 % to Ponder's mean value. This gives 0.448 + 0.060 = 0.508 %, compared with 0.504 in the present material. Crawford, Cutbush and Mollison (1953), using whole blood by a pH of 7.4 and a temperature of 23° C, got a mean value of 0.424 % for $H_{50}$. Calculated in the same way this mean value is increased to 0.524 % NaCl.

Ponder (1948 b) also gives figures of the extreme decile difference (which he called interdecile difference), which agree very well with those obtained for adults in this investigation (0.131 compared with 0.134 % NaCl).

*Fetuses*

The osmotic resistance of red cells from 13 fetuses varying in length between 13 and 28 cm (duration of pregnancy 14–21 weeks) was investigated. The main part of the mean percentage hemolysis curve was shifted to the left of the adult normal material, i.e. the majority of these fetal red cells

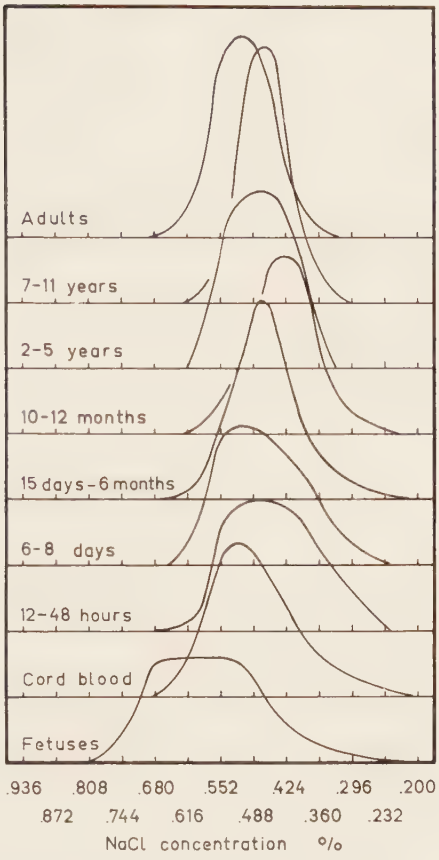



Fig. 8. Osmotic resistance distribution of the red cells at different ages. The curves were obtained by differentiation of the percentage hemolysis curves. The different curves enclose the same total area.

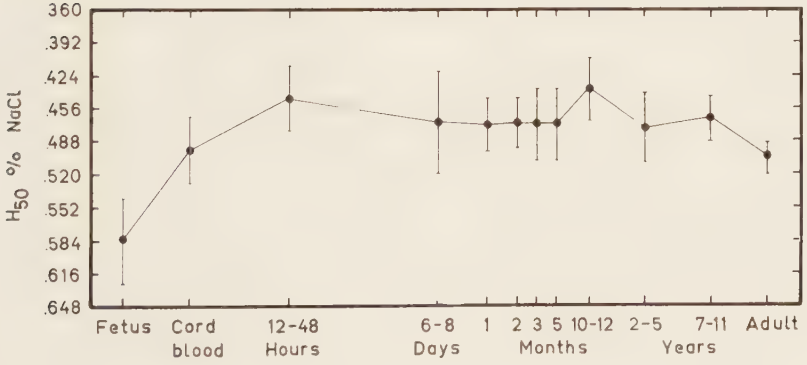
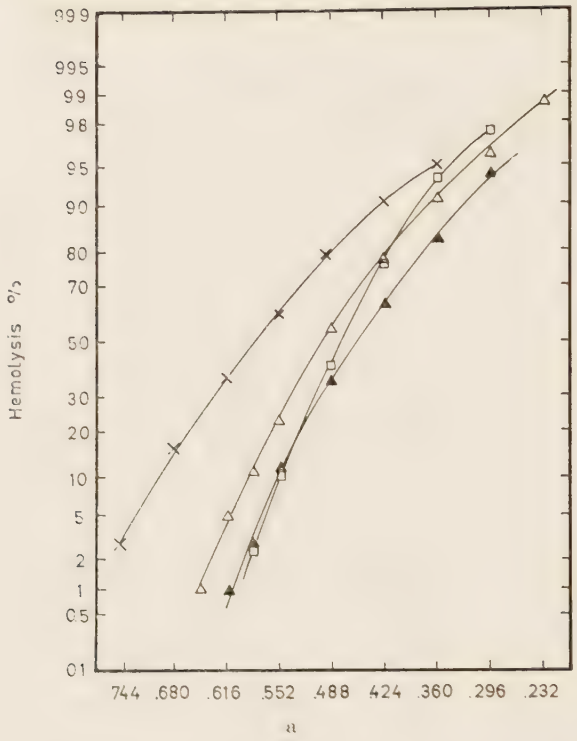
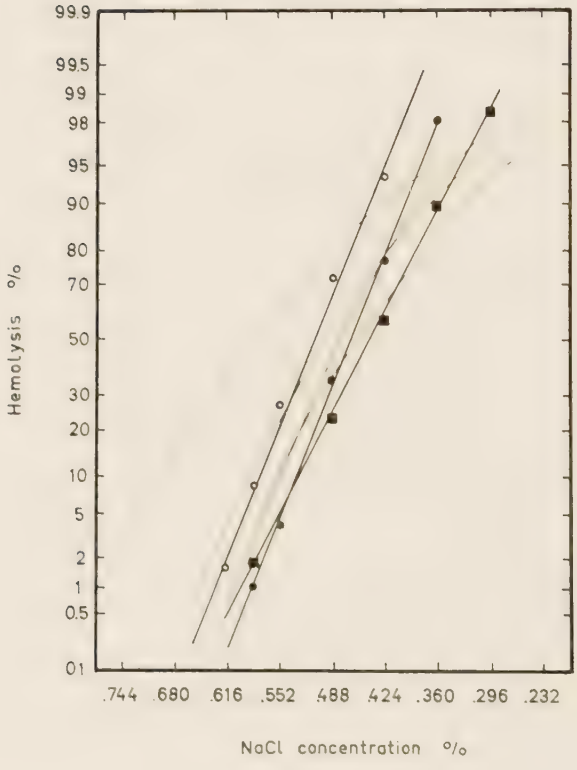



Fig. 9. Mean osmotic resistance ($H_{50}$) at different ages.




a




b

Fig. 10. Probit curves showing the degree of hemolysis of the red cells at different ages and at different NaCl concentrations. In Fig. 10 a the mean percentage hemolysis curves are given for fetuses (×), newborn infants (cord blood) (△), infants aged 12–48 hours (▲), and infants aged 15 days to 6 months (□). In Fig. 10 b the mean percentage hemolysis curves are given for infants aged 10–12 months (■), children aged 7–11 years (●), and adults (○). The curves of Fig. 10 a are drawn in thin lines for comparison.

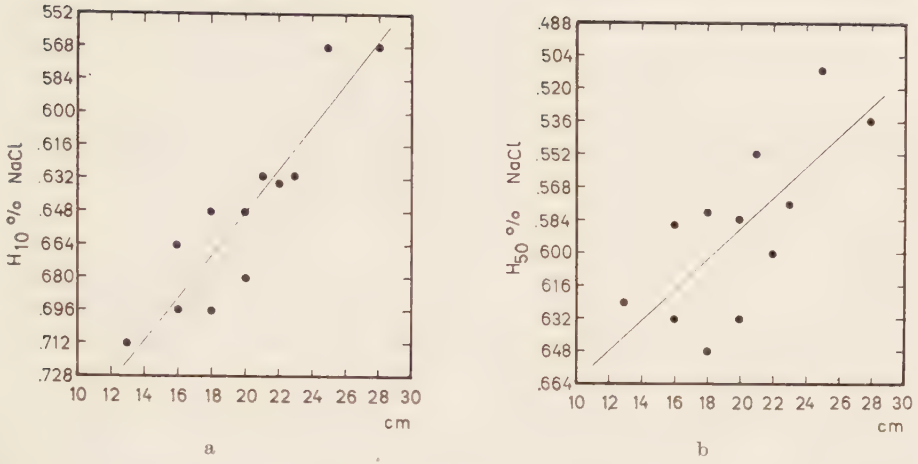



Fig. 11. Variation of the osmotic resistance expressed as $H_{10}$ (Fig. 11a) and $H_{50}$ (Fig. 11b), with the length of the fetuses.

was much less resistant than the adult red cells (Fig. 7a). The difference between the means of $H_{50}$ of the fetal and adult red cells was real (Table 1, Fig. 9). The resistance distribution curve of the fetal red cells was broad and flat (Fig. 8) and the probit line was curved (Fig. 10a). The span of hemolysis (extreme decile difference) was much greater than in the adult red cells.

The mean percentage hemolysis curve was totally shifted to the left of the cord blood curve (Fig. 7a). Of the individual curves 11 had their most resistant part within the limits of the cord blood material. The curves of the two oldest fetuses, on the other hand, were totally situated within these limits. The resistance distributions of the last two fetuses were also practically identical with the distribution in cord blood, while the others showed very skew or extremely flat distributions. In one case the distribution was bimodal.

Fig. 11 shows also that the individual values of $H_{10}$ and $H_{50}$ tended to decrease with the increasing length of the fetus. The coefficient of *regression* was found to be $-0.0104 \pm 0.0015$ for $H_{10}$ and $-0.0075 \pm 0.0019$ for $H_{50}$ (note the direction of the ordinate). Thus there is in both cases a safe covariation between the osmotic resistance and the length of the fetus, the osmotic resistance increasing with the increasing length of the fetus. The relation, however, is probably not a straight line. The same tendency was found by comparison with the menstrual age.

*Cord blood*

The cord blood of 31 *full-term* infants was investigated. The mean percentage hemolysis curve of these late fetal red cells was situated in the

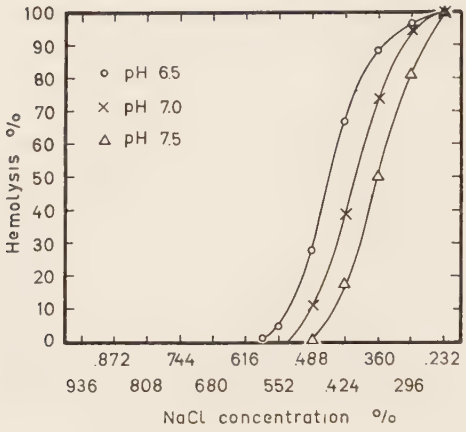



Fig. 12. Degree of hemolysis of the cord blood red cells of the same blood at different NaCl concentrations and at different pH.

neighbourhood of the corresponding adult curve (Fig. 6). The mean values of $H_{50}$ of these curves were not convincingly separated (Table 1, Fig. 9). The curve was also sigmoid, but its general slope differed from the adult curve. The cord blood curve started a little to the left of the adult curve, but the greatest part ran to the right. This indicates a few cells with a lower resistance and a great amount of cells with a higher resistance than the adult red cells. The distribution curve was skew (Fig. 8). The span of hemolysis was greater than in the adult curve. The range of normal variation in osmotic resistance of late fetal red cells seems to be greater than in the adult material (Fig. 6).

Also *washed* red cells from cord blood and adults showed the same characteristic differences.

The cord blood of two *premature* infants (born 5 and 7 weeks before term respectively) was also investigated. The main part of their percentage hemolysis curves was within the normal limits of the newborn, full-term infants. A small number of the red cells, however, was less resistant than the cells from cord blood of the full-term infants.

In accordance with the situation in adults a *change in pH* results in a practically parallel shift of the percentage hemolysis curves of cord blood red cells (Fig. 12).

### *Infants aged 12–48 hours*

The mean percentage hemolysis curve of 20 *full-term* infants aged 12–48 hours was shifted to the right of the corresponding cord blood curve indicating an increased osmotic resistance (Fig. 7). The difference between the means of $H_{50}$ was real (Table 1, Fig. 9). The general shape of the curve was

similar to the cord blood curve. The span of hemolysis was practically the same as in the cord blood.

Two *premature* infants, born 6 and 7 weeks before term respectively, had their percentage hemolysis curves to the left of the full-term range. Neither the distribution curves nor the span of hemolysis differed from those of the full-term infants.

### *Infants aged 6–8 days*

In 11 *full-term* infants aged 6 to 8 days the mean percentage hemolysis curve had shifted to the left and was situated between the cord blood curve and the 12–48 hours curve. The mean value of $H_{50}$, however, was not separated from the corresponding mean value of the infants aged 12–48 hours, nor from the mean value of the infants aged 15 days to 6 months (Table 1, Fig. 9). The frequency distribution showed a slight positive skewness (Fig. 8). The span of hemolysis was practically the same as in the cord blood curve.

Two *premature* infants born 9 and 4 weeks before term, respectively, had their percentage hemolysis curves within the ranges of the full-term material. Neither the distribution curves nor the span of hemolysis differed from those of the full-term infants.

### *Infants aged 15 days to 6 months*

Forty-eight *full-term* infants aged from 15 days to 6 months were investigated. They were at first divided into four groups (15–45 days, 46–75 days, 76–105 days, and 4–6 months, Table 1, Fig. 9). Since no remarkable differences in osmotic resistance were revealed between these four groups they were subsequently treated as a single group.

The whole mean percentage hemolysis curve of this group was situated to the right of the corresponding adult curve and the main part also to the right of the cord blood curve, indicating that a great part of the red cells of these infants was more resistant than both adult and cord blood red cells (Fig. 10a). The mean value of $H_{50}$ in these infants was separated from the corresponding means in adults, in cord blood, in infants aged 12–48 hours and 10–12 months (Table 1, Fig. 9). The resistance distribution curve was slightly skew (Fig. 8 and 10a). The span of hemolysis was definitely lower than during the first eight days of life.

In four *premature* infants, aged 16 to 33 days and born at least 4 weeks before term, the percentage hemolysis curves were situated within the ranges of the full-term material, but the distribution curves were more like those of the earlier age periods and the span of hemolysis still showed the high values found at birth.

*Infants aged 10–12 months*

The mean percentage hemolysis curve of 11 infants, aged from 10 to 12 months, was partly shifted to the right of all other curves indicating a great number of cells with high osmotic resistance (Figs. 7b and 10b). The nearest curve was that of the infants aged 12–48 hours; their means were not significantly separated. In contrast to the curved probit line of the earlier age periods the probit curve had become a straight line and the resistance distribution had thus become practically normal (Figs. 10b and 8). The span of hemolysis was less than during the first eight days of life and the same as during the age period of 15 days to 6 months.

In looking for an explanation of this remarkable increase in osmotic resistance the experimental conditions have been controlled scrupulously. The individual osmotic resistance tests have been compared with 2–5 tests of the same day from other age groups. There were no indications of any systematical experimental errors.

*Children aged 2–11 years*

The mean percentage hemolysis curve of 26 children, aged from 2 to 11 years, was situated to the right of the adult and the cord blood curves, and to the left of the curves of infants aged 12–48 hours and 10–12 months (Fig. 7b and 10b). The mean value of $H_{50}$ was significantly separated from all other mean values of $H_{50}$, except from that of the infants aged from 15 days to 6 months (Table 1, Fig. 9). The frequency distribution of the resistance was normal (Fig. 8). In the age group 2–5 years the span of hemolysis was greater than in the age group 7–11 years, in which it was about the same as in the adult material (Table 1). A great number of red cells of this age were thus more resistant than the adult and the cord blood cells.

CHAPTER III

# Investigations on the Significance of the Osmotic Resistance Tests

## Earlier Investigations

The interpretation of the results of osmotic resistance tests offers difficulties and requires knowledge of the different factors that are, or are supposed to be, connected with the osmotic resistance. Before discussing the results of the osmotic resistance tests these factors will be reviewed and some experiments concerning this problem will be reported.

The osmotic resistance of a red cell is closely related to its *initial shape* and volume (Haden 1934; Dacie and Vaughan 1938; Ponder 1948a). According to the description of the hemolysis in hypotonic saline solutions (p. 14) it is evident that a flat cell can take up more water than a spherical cell before reaching the critical volume. The hemolysis of a flat cell thus takes place in a lower saline concentration, i.e. a flat cell is more resistant than a more spherical cell. In accordance with this fact the spherocyte index, which is an approximate but fairly good measure of the degree of spherocytosis, is said to be significantly correlated to the median red cell resistance (Haden 1934; Dacie and Vaughan 1938). The shape of a red cell may be determined not only by its inherent properties but also by *extrinsic influences* that may alter the dissociation of the hemoglobin and the interior osmotic pressure. Methodological factors discussed above, such as carbon dioxide content, pH, temperature and oxygenation, exhibit their effects on the osmotic resistance in this way. Jacobs and Parpart (1931) have shown that the effect of these factors must at least largely be attributed to their influence on the base-binding power of the hemoglobin. By an increased carbon dioxide content or an otherwise lowered pH the base-binding capacity of the hemoglobin is lowered, the interior ion concentration is increased, the osmotic pressure is increased, the cell takes up water and swells, and its osmotic resistance is decreased. The same is the case when the temperature or the oxygenation is lowered.

The osmotic resistance, however, is also influenced by factors that do not affect the initial shape of the red cell. In red cells that are imperfect osmometers the *critical volume* differs from that of a perfect osmometer. If the

critical volume is smaller the osmotic resistance is decreased; if it is greater, which is more unusual, the resistance is increased. The imperfect osmometers thus show changes in osmotic resistance independent of their initial shape. Factors such as the type of anticoagulant and the type of hypotonic media influence the osmometer function; whether it happens by affecting the cell membrane or the state of the hemoglobin is not known. The special intrinsic cell properties in sickle cells and thalassemia cells also affect the osmometer function, and as a result of this the osmotic resistance is changed in a way that is not explained by a shape factor. In congenital hemolytic anemia the osmotic resistance may remain decreased though the cells after splenectomy return to a normal thickness (VAUGHAN 1937).

Whether the osmotic resistance of a red cell is correlated to its *age* has been very much discussed. The resistance distribution to the saline concentration is a statistical distribution. If the age of the red cells were the *only* factor responsible for changes in osmotic resistance, the resistance distribution should have the same shape as the age distribution, i.e. rising or declining continuously with decreasing NaCl concentration. GERBSTÄDT (1952) has recently discussed these problems in detail. Whether the age is one of many factors is still an unsolved problem. GERBSTÄDT and KOTULEK (1953) concluded that the osmotic resistance of red cells is independent of their age.

In all discussions of this subject the *reticulocytes* have been given special attention, since their approximate age is known and they are easy to separate. There is, however, considerable divergence of opinion as to their osmotic resistance. Some authors have stated them to be more resistant than the mature cells (STEPHENS 1940), others have found them to be less resistant (GOLDBLOOM and GOTTLIEB 1929), and others have found them to be of the same resistance (WITHBY and HYNES 1935). The disagreement is at least partly explained by the inaccurate methods used. One of the most detailed investigations on this problem is that of GERBSTÄDT and KOTULEK (1953). They separated the reticulocytes of rabbits (using the lower specific gravity of reticulocytes) and tested their resistance in hypotonic NaCl solutions by staining and counting the unhemolysed cells. It was shown that the osmotic resistance of the reticulocytes described a sigmoid percentage hemolysis curve situated at a lower saline concentration than the total population. The resistance distribution of the reticulocytes was practically normal and of the same shape as the distribution of the total population, only displaced in the direction of increased resistance. Using radioactive iron, CRUZ *et al.* (1941) and STEWART *et al.* (1950), however, were able to show that in dogs the *newly formed red cells* during their first 3–4 days in the circulation were less resistant than those of the general population.

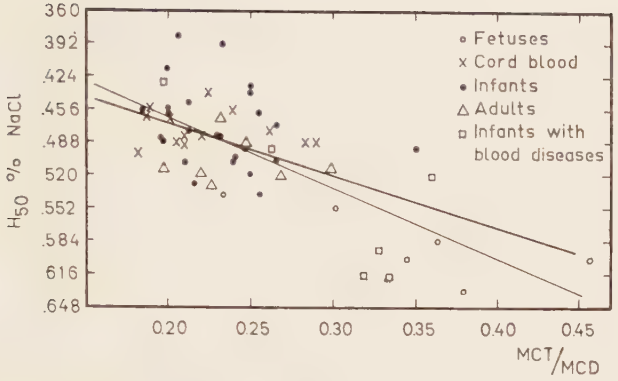



Fig. 13. Relation of the mean osmotic resistance ($H_{50}$) to spherocyte index (MCT/MCD).

With regard to the very *old cells* Cruz *et al.* (1941) stated that there were indications of their being more resistant than the mean age group. Stewart *et al.* (1950) on the other hand could find no distinctive behaviour of the old red cells.

## Experiments Regarding the Shape Factor

In order to elucidate the possible role of a shape factor in the present material, the spherocyte index was calculated in 54 cases including fetuses, newborn infants, infants, children, and adults. The result is seen from Fig. 13, where the values of $H_{50}$ are plotted against the spherocyte index. The regression line cannot be calculated by the method of least squares, because there are errors in both variables. Therefore the method of Bartlett (1949) was used. The coefficient of *regression* was found to be $0.50 \pm 0.091$. Thus there is a safe covariation between the values of $H_{50}$ and the spherocyte indices, the indices increasing with decreasing resistance. If three children with congenital hemolytic anemia, one child with acquired hemolytic anemia, one with hypersplenism and one with celiac disease were added to the normal material, the coefficient of regression would be $0.66 \pm 0.087$. If the fetuses and the sick children are excluded, the coefficient of regression would not be significantly separated from zero.

## Experiments Regarding the Age Factor

The cord blood of two infants was examined with respect to the percentage of *reticulocytes* of the unhemolysed red cells during an osmotic resistance test. The result is seen from Table 2. With increasing degree of hemolysis the unhemolysed reticulocytes, when expressed as the percentage of the initial number at first increased above the initial level and then decreased. The

Table 2

| NaCl % | Unhemolysed red cells % | Reticulocytes of unhemolysed red cells % | Number of unhemolysed reticulocytes, % of the initial value | Hemolysis, % | |
|---|---|---|---|---|---|
| | | | | Red cells | Reticulocytes |
| 0.968 | 100 | 2.8 | 100 | 0 | 0 |
| 0.584 | 88 | 3.3 | 104 | 12 | 0 |
| 0.552 | 75 | 8.2 | 221 | 25 | 0 |
| 0.488 | 46 | 7.8 | 129 | 54 | 0 |
| 0.424 | 23 | 11.1 | 93 | 77 | 7 |
| 0.968 | 100 | 1.2 | 100 | 0 | 0 |
| 0.744 | 100 | 1.0 | 83 | 0 | 17 |
| 0.648 | 100 | 2.6 | 217 | 0 | 0 |
| 0.584 | 99 | 6.5 | 533 | 1 | 0 |
| 0.520 | 86 | 5.4 | 383 | 14 | 0 |
| 0.456 | 48 | 3.5 | 142 | 52 | 0 |

increase may be explained by more red cells becoming stainable with increasing volume or increasing crenation of the cells. It is also possible that the swollen red cells show another distribution on the slide than the untreated cells. The reticulocytese did not significantly decrease below the initial level.

The reticulocytes have a lower specific gravity than the mature cells. It is thus possible to obtain samples of red cells with a high percentage of reticulocytes by collecting the uppermost third of centrifuged blood samples. The osmotic resistance of such an upper third was determined in two newborn infants and one adult. The bottom third of the centrifuged sample was tested for comparison. Fig. 14 illustrates the results. In one of the newborn infants the specific lighter cells were markedly more resistant, in the other newborn infant the difference was slight but quite apparent. In the adult only a very slight difference was noticed between the upper and the lower portion, possibly due to a low percentage of reticulocytes.

The bone marrow contains a relatively greater number of *young cells* (immature and mature) than does the peripheral blood. Differences between the bone marrow blood and the peripheral blood may thus be ascribed to the higher content of young cells in the bone marrow. To get an idea of the osmotic resistance of the young red cells, osmotic hemolysis curves from these two sources were compared. Venous blood and bone marrow blood was obtained from a microcephalic infant aged two years. The result is illustrated in Fig. 15. Evidently the young cells were less resistant to hypotonic NaCl solutions than the general population of peripheral red cells.

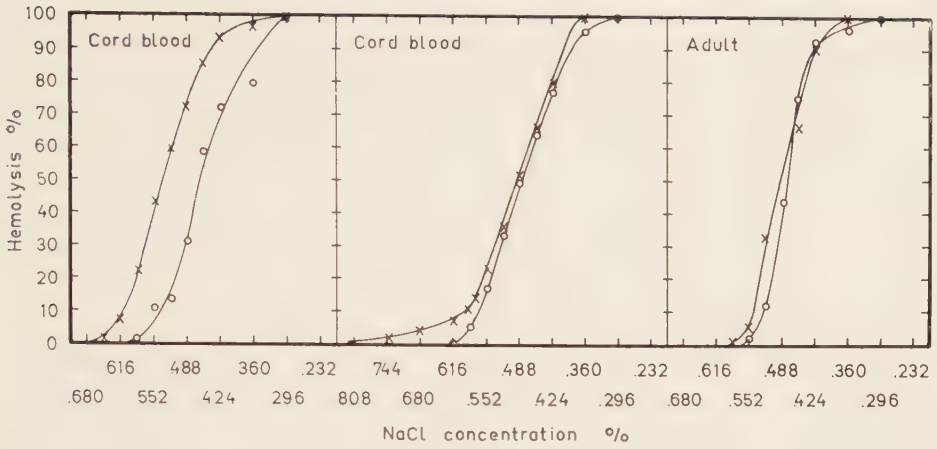



Fig. 14. Osmotic resistance of washed red cells from the upper (○) and the lower (×) third of centrifuged blood samples of two newborn infants (cord blood) and one adult.

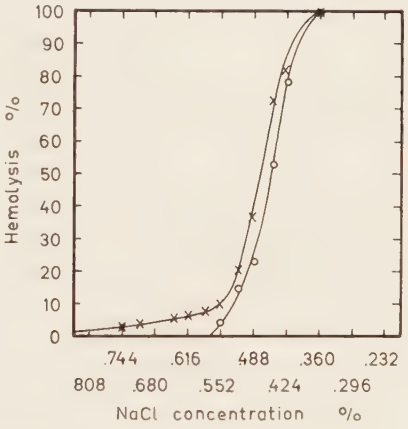



Fig. 15. Osmotic resistance of the red cells from venous blood (○) and bone marrow blood (×) of an infant aged 2 years.

The fetal red cells that contain fetal hemoglobin disappear successively during the first months of life. During the same time only a few of the newly formed red cells contain fetal hemoglobin. Thus it seems likely that during the first months of life *old red cells* are overrepresented among the red cells that contain fetal hemoglobin.

In five infants, two newborn, one aged $1^1/_2$ month, one aged 4 months and one $5^1{}_2$, the following experiment was performed. The fetal hemoglobin concentration of the general red cell population was determined. About one half of the red cells was then hemolysed in a hypotonic NaCl solution. The unhemolysed cells were collected, and the fetal hemoglobin concentration of the unhemolysed cells was determined. The results are given in Table 3. At birth there was no difference in fetal hemoglobin concentration between the red cells of

TABLE 3

| Age of infants | Fetal hemoglobin, % | |
|---|---|---|
| | General population | Most resistant population |
| Newborn . . . . . | 70 | 68 |
| Newborn . . . . . | 66 | 65 |
| 1 ½ month . . . . . | 58 | 63 |
| 4 months . . . . . | 7 | 22 |
| 5 ½ months . . . . . | 10 | 17 |

the general population and the osmotically most resistant ones. With increasing age of the infant, however, there was a higher percentage of fetal hemoglobin in the most resistant red cells. The most likely explanation seems to be that there is a comparatively greater number of old fetal cells in the most resistant red cell population than in the general population.

## Discussion of Chapters II and III

Evidently it is difficult to interpret the results of osmotic resistance tests even when they are supplemented with special investigations concerning the main factors that influence the osmotic resistance.

It has not been considered necessary to control the *osmometer function* because the investigations of GUEST (1948) show that red cells from infants, children and adults are nearly perfect osmometers under the experimental conditions used.

The role of a *shape factor* in the present material is difficult to establish. Fig. 13 at first gives the impression that a shape factor is at work. It must be remembered, however, that the red cells of the fetuses and the sick children mainly determine the regression lines obtained. In the healthy children and in the adults it was not possible to show that the osmotic resistance varied with the degree of spherocytosis. According to the generally accepted view of the process of osmotic hemolysis, however, it is very probable that the shape of the normal red cells also influences their osmotic resistance. The determination of MCT is based on the packed red cell volume and the red cell count, the enumeration of the latter is subject to great errors. Also, the formula for calculating the MCT is rather uncertain. A great error is thus to be expected in MCT MCD and it seems probable that only great differences in the degree of spherocytosis will be revealed with the methods used. In any case it seems *permissible to interpret the changes in osmotic resistance as depending, at least partly, on variations in red cell shape.*

The possible role of an *age factor* is also difficult to establish. The above

investigations on reticulocytes, as well as those of GERBSTÄDT and KOTULEK (1953), show that reticulocytes as a whole are more resistant than the general red cell population. The above investigations on bone marrow and the investigations of CRUZ *et al.* (1941), and STEWART *et al.* (1950), on the other hand, show that the very young cells are less resistant than the cells of the general population. At first these statements seem contradictory. The differences are probably not to be sought in unsatisfactory methods. One explanation may be that the reticulocytes have a longer maturation time in the circulating blood than the three to five days given by most authors. Another explanation, however, seems more likely. Some authors have concluded that all red cells pass out of the bone marrow as reticulocytes (SEIP 1953), others have claimed that most red cells are fully matured when they leave the marrow (YOUNG and LAWRENCE 1945). WINTROBE (1951) states that the latter view is the more plausible. If this is the case, different types of cells may have been tested by the different methods—all newly formed cells with the radioactive iron method, and only the immature, stainable reticulocytes with the other one. The results rather seem to support the view that red cells leave the bone marrow both as reticulocytes and as young mature cells. Because the decreased resistance of the young cells is the only one visible by the radioactive iron method, the mature cells are probably in the majority.

With regard to the results of CRUZ *et al.* (1941) and to the above investigations on the concentration of fetal hemoglobin in different cells, it is probable that the oldest cells as a whole have an increased resistance in comparison with the general red cell population.

Obviously there is no continuous and regular correlation between the red cell age and the osmotic resistance, even if it is shown that the age of the red cells influences the osmotic resistance. In any case it is not possible to draw any conclusions from the osmotic resistance as to the age of the different cells. We may conclude, however, that *newly formed mature cells have a lowered osmotic resistance during the first 3–4 days, while most of the reticulocytes and the very old cells seem to be more resistant than the general red cell population.*

There are also more general conclusions to be drawn from the variations in osmotic resistance. PONDER (1948a) states that "it is probably safe to interpret changes in the form of the resistance distribution as evidence of changes in the composition of the red cell population". This statement implies that the osmotic resistance test, expressed as resistance distribution, is a sensitive method for discovering changes in the red cell population during different conditions, for instance at different ages of life. PONDER (1948b) has shown how bimodalities appear after hemorrhagic and hemolytic episodes. In addition, it ought to be stressed that the method, from a statistical point

of view, has the advantage of being based on millions of red cells, which makes it much more reliable than, for instance, measurements of red cell diameters.

*The characteristic variation of the osmotic resistance at different ages must thus be regarded as an indication of variations in the composition of the red cell population or in the red cell environment.* It is very difficult to put these variations of resistance in direct relationship with other parallel changes in red cell physiology. Some suggestions, however, seem possible.

The *fetus material* of this investigation covers the fetal age from $3\,^1/_2$ to $5\,^1/_4$ months, a time during which the hepatic period of erythropoiesis dominates and the mesoblastic erythropoiesis has just ceased (Fig. 1). The change in osmotic resistance that occurs during this time is a gradual increase in resistance, the red cells at the beginning of the fifth month being within the limits of the normal cord blood values (Fig. 11). In this situation it would seem that *the majority of the less resistant cells arise from the mesoblastic centres of erythropoiesis.* The shape of the resistance distribution is quite in accordance with the red cells belonging to two different populations with different resistance distributions (Fig. 8). The similarity between the resistance distribution of the two oldest fetuses, of the premature and of the full-term infants at birth suggest that only comparatively small changes take place during the last four months of gestation. *It seems likely that cord blood on the whole can be considered representative of the osmotic resistance during the last months of gestation.* The remarkable difference in osmotic resistance between the mesoblastic and the hepatic red cells indicates quite different physiological properties of these two types of cells.

Many factors have to be considered when the characteristic shape of the *osmotic resistance distribution of cord blood* is discussed.

Because the washed fetal and adult red cells show the same characteristic differences as the unwashed cells, *the differences must be attributed to red cell factors only*, not to any plasma constituents.

*Change in pH* means a practically parallel shift of the percentage hemolysis curve (Fig. 12). One small difference, however, is noticed. When the pH is lowered the most resistant part of the curve becomes more extended and the skewness of the distribution thus becomes more apparent. In this way the pH used here has contributed to an increased sensitivity of the method.

During the first week of life the young cells, both from the bone marrow and the liver, are numerous in full-term infants. Thereafter the signs of high erythropoietic activity disappear and the number of reticulocytes and other young cells decreases to about adult values. In premature infants the high content of young cells persists for a longer time (Magnusson 1935). The young cells have been shown to consist of two osmotically different groups,

one more and one less resistant than the general red cell population. Because the span of hemolysis, expressed as the extreme decile difference, strikingly diminishes during the second week of life, it seems rather probable that *the excess of young cells is responsible, at least in part, for the increased span of hemolysis* most characteristic of the osmotic resistance of the red cells at birth. The fact that the span of hemolysis decreased later in premature infants than in full-term infants supports this opinion.

In this connection the possible role of the *fetal hemoglobin* has to be considered. Because the *isoelectric point* of the fetal hemoglobin is lower than that of the adult hemoglobin, the base-binding capacity of the fetal hemoglobin is greater than that of the adult hemoglobin at the same pH. If it is assumed that the adult and the fetal red cells have the same mean cell hemoglobin concentration, this difference in IP means that the osmotic pressure of the fetal red cells is less than that of the adult red cells. The osmotic resistance of the fetal red cells thus ought to be increased. This increase, however, cannot be great and can hardly explain the observed differences in osmotic resistance between cord blood and adult red cells.

At the same pH the degree of *hydration of the fetal and the adult hemoglobin* is not the same, depending on their different IP. Whether the difference in hydration influences the osmotic resistance is not known, but a small influence cannot be excluded.

The pronounced *anoxemia and acidosis* regularly found at birth tend to increase the water content of the red cells in vivo. When tested under these environmental conditions the osmotic resistance ought to be lowered. By the osmotic resistance test in NaCl, however, these factors are compensated by the oxygenated and buffered NaCl solutions used. It thus seems unlikely that the differences between the osmotic resistance of the late fetal red cells and the adult cells are to be looked for in these factors.

The strikingly *great variation in osmotic resistance of cord blood cells from different infants* (Fig. 6) is in good accordance with the varying proportions of liver erythropoiesis and bone marrow erythropoiesis found at birth in different infants (Knoll 1950).

*The increase in osmotic resistance after birth* is significantly demonstrated already in *the age group 12–48 hours* (Fig. 7 a). The earlier hours seem to be a transitional period. Crawford, Cutbush and Mollison (1953) found no significant difference between the mean cell resistance ($H_{50}$) at birth and in infants aged 2–24 hours ($P$ 0.07). Judging from Waugh, Merchant and Maughan (1939) the maximal increase is reached on the fourth day. The next six to ten days, again, are a transitional period during which the osmotic resistance increases until a level is reached where it remains, with one exception, throughout childhood (Fig. 9). That the age 6–8 days is a transi-

tional period is also demonstrated by the great individual variation in osmotic resistance ($\sigma$ ($H_{50}$) = $\pm$ 0.051).

The postnatal increase in osmotic resistance is difficult to interpret. It appears very suddenly and may hardly be indicative of changes in the composition of the red cell population. The resistance distribution is also practically the same as that found at birth. *It seems more likely that the shift of the curve is connected with the sudden changes in metabolism known to take place after birth.* Due to reasons mentioned above it is not probable that the changes in oxygenation or in pH in themselves have any influence on the osmotic resistance. The shift from a relatively anaerobic to a more aerobic carbohydrate metabolism (RÄIHÄ 1940; WILSON, REARDON and MURAYAMA 1948) with a parallel completion of the enzyme systems, however, might well be of significance in this connection. It may also be worth mentioning that in premature infants the postnatal increase in osmotic resistance seems to develop more slowly than in full-term infants. Also, the striking *loss of body water* at this time must be taken into account. The loss of water is thought to be a loss both of extra- and intra-cellular water. If the red cell content of water also decreased without a parallel loss of electrolytes, an increased osmotic resistance would be the consequence. This factor would also explain the decreasing resistance at the end of the first week.

As to the nature of the physiological anemia developing during the first months of life no new information has been obtained by this investigation on the osmotic resistance during the neonatal period.

At present it seems *practically impossible to give a convincing explanation of the increased osmotic resistance of the red cells of the infants aged from 15 days to 11 years.* The microcytes of hypochromic anemia have an increased osmotic resistance and it is debatable whether the microcytes normally seen in infants from the third month onwards have the same properties. The increased osmotic resistance of the microcytes of hypochronic anemia seems to be due to their low mean cell hemoglobin concentration. The microcytes of the normal child, however, have a normal mean cell hemoglobin concentration and thus it seems very unlikely that the microcytosis in itself explains the increased osmotic resistance during childhood. The microcytosis and the increased osmotic resistance may only be parallel changes indicating that the red cells of infants differ in many respects from the adult red cells.

*The remarkable additional increase in osmotic resistance in infants aged 10–12 months* is equally inexplicable (Fig. 9). It seems worth mentioning, however, that the mean cell hemoglobin concentration, the mean cell volume, and the mean cell diameter reach their lowest values at this time. In any case, the increased osmotic resistance is *an indication of a definite change in the red cell population at this age.*

CHAPTER IV

# On the Kinetics of Water Permeation of the Adult and the Fetal Red Cells

The rate of entrance of water into a red cell in hypotonic NaCl solutions depends not only on the difference in osmotic pressure between the red cell interior and the surrounding hypotonic media, but also on the area of the red cell surface and the properties of its membrane. As a measure of the flow of water into a red cell the *permeability coefficient*, $P_w$, is often used. It is defined as *the quantity of water that will cross a unit area of the red cell membrane in unit time with unit difference in osmotic pressure between the two sides of the membrane.* At present the square centimeter is most commonly used as the unit of area, the cubic centimeter as the unit of volume, and the hour as the unit of time. The driving force is expressed in terms of effective concentration difference measured in moles per cubic centimeter (Jacobs 1952). The units used earlier were $\mu^3$, $\mu^2$, minutes, and atmospheres. The permeability coefficient was then called $k$. The relation between $P_w$ and $k$ is $P_w = k \times 134.4$.

The water permeability of red cells was treated mathematically by Jacobs (1932 a) who assumed the red cell to be a perfect osmometer without selective permeabilities for ions as K and Na, a situation that is true only at low degrees of hemolysis before any loss of K has occurred (see discussion p. 55). Jacobs then used the relations

$$C\,V = C_0 V_0 \tag{1}$$

$$\frac{d\,V}{d\,t} = P_w A\,(C - C') \tag{2}$$

where $C_o$ is the osmotically effective concentration and $V_o$ the osmotically effective volume of the red cell at zero time, $C$ is the concentration inside the cell at time $t$, $V$ is the osmotically effective volume of the cell at time $t$, $P_w$ is the permeability coefficient, $A$ is the area through which the entrance of water occurs, $C'$ is the concentration of the outside solution.

By solving the equation system (1) and (2) the following equation is obtained:

$$\frac{P_w A}{V_0} \cdot t = \frac{1}{C'} \left[ \left( 1 - \frac{C_0}{C} \right) + \frac{C_0}{C'} \ln \frac{1 - C'/C_0}{1 - C'/C} \right]. \quad (3)$$

For the determination of the permeability coefficient, JACOBS used distilled water ($C' = 0$) and measured the time ($t$) for 75 % hemolysis. At this time the value of $C_o/C$ was assumed to be 1.7. For adult red cells the value of $P_w$ was then found to be about 400 (JACOBS 1952).

In the units mentioned above the dimensions of $P_w$ will be $cm^4\ hour^{-1}\ moles^{-1}$. The conventional dimensions of a permeability coefficient, $P$, however, is cm $sec.^{-1}$. The reason of this discrepancy is that in equation (2) the driving force is expressed as the difference in NaCl concentration between inside and outside, i.e. osmotic pressure difference, and not as the difference in water concentration, which would be correct, because water is the permeating substance in these experiments. It would be an advantage if the permeability coefficient for water ($k$ or $P_w$) could be converted into units of cm $sec.^{-1}$. Converting the osmotic pressure into water concentration ZWOLINSKI, EYRING and REESE (1949) showed that the coefficient $k$ had to be multiplied by the factor $\frac{RT}{V_m}$, where $R$ is the gas constant, $T$ the absolute temperature and $V_m$ the molal volume of water. $\frac{kRT}{V_m}$ then is the usual permeability coefficient, and has the dimension cm $sec.^{-1}$. The two factors can be converted according to

$$P\ (cm/sec.) = k \times 7.57 \times 10^{-6} \times T\ (°K). \quad (4)$$

If $k$ is exchanged for $P_w$ and the temperature used in the present investigation (20° C) is inserted, the expression will be

$$P = \frac{P_w}{134.4} \times 7.57 \times 10^{-6} \times 293 = P_w \times 16.5 \times 10^{-6}\ \text{cm sec}^{-1}. \quad (5)$$

## Earlier Investigations

Although valuable information about the red cells can be obtained by permeability studies, the fetal red cells have attracted very little interest with respect to the rate of entrance of water from a surrounding hypotonic NaCl solution.

MOSER and CNYRIM (1950) studied the osmotic swelling of the red cells of infants of different ages after "accumulating" the cells with glucose. The

experimental conditions seem to have been satisfactory. They treated the results by making "Volumzuwachskurven" (curves of the volume increase) at different times of the experiments. The "Volumzeitkurven" (curves of the rate of volume increase) that give the most detailed information about the rate of swelling were incompletely analysed. They stated that red cells of newborn infants seemed to swell more rapidly than cells of infants aged one month and of children aged 2 years, but a little slower than adult red cells. They did not calculate any figures of permeability. Their interpretation of the results is difficult to follow and is also obscured by the glucose accumulation. The relatively rapid swelling of the red cells of newborn infants was ascribed to the acidosis occurring at this age. The slow swelling of the red cells of older infants and young children was ascribed to a high number of flat cells during these ages. The authors do not believe that differences of the membranes of different types of cells are responsible for the differences in swelling. The only conclusion which can safely be drawn from their results is that there are distinct differences in the osmotic properties of the red cells of infants and adults.

Widdas (1951) reported investigations on the osmotic properties of sheep erythrocytes and could show that the rate of entrance of water was much slower in fetal than in maternal sheep red cells. He also showed that there was a progressive increase in water permeability with increasing fetal age. The permeability differences were attributed to differences in the membrane properties of fetal and adult red cells and it was suggested that differences in surface pressure might be the explanation. Widdas did not calculate the permeability coefficients.

## Methods

The *rate of entrance of water into a red cell in hypotonic NaCl solutions* can be determined by an electrophotometric method similar to the method described by Wilbrandt (1938). If a *red* filter is used, the light is scattered when it passes through the red cell suspension; only little is absorbed. This scattering depends on the number, the size and the shape of the particles in the solution. When the red cells change shape, i.e. swell or crenate in the hypotonic NaCl solutions the refraction of the light is changed, so that the light scattering is decreased and the transmission is increased (cf. the discussion of Wilbrandt 1938; Ponder 1948a, p. 76; Loeschcke and Wever 1951; and Teorell 1952).

The relation between the transmission and the volume of the red cells was a straight line until the red cells started to hemolyse (Fig. 16). In cord blood the slope of the line was more acute to the volume axis than in adult blood. This probably depended on the different initial shape of the cord blood cells and the adult cells.

When hemolysis starts the number of red cells begins to diminish and an additional increase in transmission, proportional to the degree of hemolysis, takes place. The

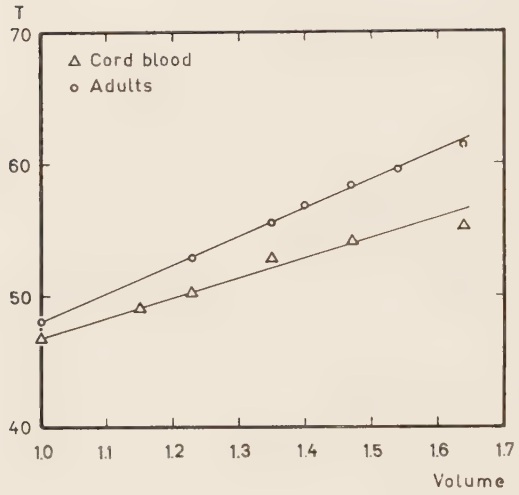



Fig. 16. The transmission plotted against the relative volume of the red cells in different prehemolytic NaCl solutions.

rate of swelling and the rate of hemolysis are thus measures of the rate of the water entrance.

The photoelectric colorimeter described above (p. 20) was used. The red filter did not transmit light of wave lengths below 580 mμ. The current of the photocell operated a Moll Micro Galvanometer (No. A 23, Kipp & Zonen, Delft), the deflection of which was traced on a photo recording paper (width 120 mm) in a drum camera. A cuvette of 1.00 cm length was used. In order to increase the sensitivity for volume changes both the test and the standard cuvette were placed at a distance of 2 cm from the photocell.

The series of hypotonic NaCl solutions was the same as in the osmotic resistance tests and the solutions were prepared and handled in the same way. The final concentrations varied from 0.968 % to 0.200 %. Buffered red cell suspensions were prepared as in the osmotic resistance tests (p. 19).

At the start of a test 3 ml of the hypotonic NaCl solution was poured into the test cuvette. Thereafter 0.75 ml of the red cell suspension was rapidly injected into the hypotonic NaCl solution. The pH was 7.0 and the final concentration of red cells

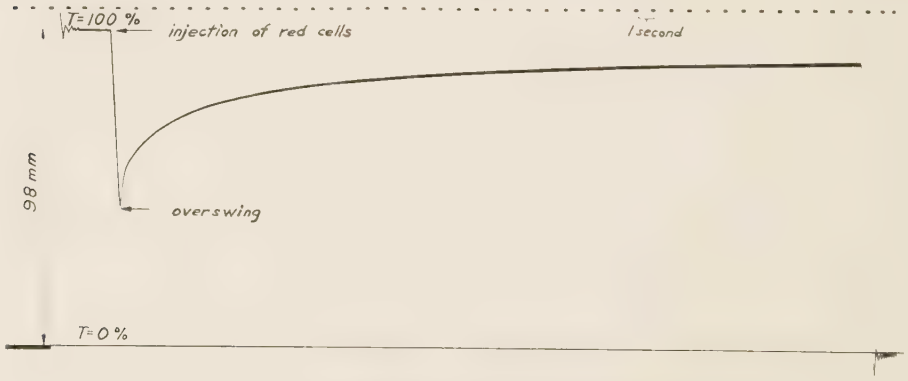



Fig. 17. The rate of hemolysis of adult red cells in 0.232 % NaCl.

1/200. Continuous cautious stirring with a glass propeller was maintained throughout the experiments. The temperature varied between 19° and 21° C. A flashing light recorded the time each second. The experiments were run until a practically stable degree of transmission was reached.

A typical record is shown in Fig. 17. The overswing of the galvanometer is rapid and not disturbing. The transmission reading can be transformed to per cent hemolysis by a calibration curve made of samples of known degree of hemolysis.

The *blood samples* were obtained as above (p. 18).

Concerning *material* see p. 24.

## Analysis of the Experiments

The results were analysed mainly following WIDDAS (1951). If the symbol $t_h$ represents the time the cell needs to reach its critical volume and $C'_h$ is the inside concentration at this time, the equation (3) can be written

$$\frac{P_w A}{V_0} \cdot t_h = \frac{1}{C'} \left[ \left( 1 - \frac{C_0}{C_h} \right) + \frac{C_0}{C'} \ln \frac{1 - C'/C_0}{1 - C'/C_h} \right]. \tag{6}$$

In this equation the function of the right hand side depends on the outside concentration ($C'$) and on the inside concentration ($C_h$) at time $t_h$, because $C_o$ is constant throughout. The equation (6) can thus be rewritten

$$\frac{P_w A}{V_0} t_h = f(C'; C_h). \tag{7}$$

If $f(C''; C'_h)$ could be calculated it would give a straight line passing through the origin and having a slope of $P_w A / V_o$ if plotted against $t_h$. *The value of* $P_w A / V_o$ *thus is a measure of the permeability* and has the dimensions $cm^3$ $hour^{-1}$ $moles^{-1}$. If we know the area ($A$) and the initial volume ($V_o$) the permeability coefficient can be calculated.

The time a red cell needs to reach its critical volume in different outside concentrations is calculated in the following way:

In the most hypotonic NaCl solutions all red cells are hemolysed. In the other solutions a certain number of red cells remains unhemolysed (Fig. 18). In all solutions the transmission increases until a reasonably steady state level, $T_s$, is reached, which is dependent on the outside concentration. At this level, $T_s$, some red cells have reached their critical volume ($V_h$). The inside hemolytic concentration ($C'_h$) of these particular cells is then the same as the outside concentration ($C'$). In a less hypotonic solution these cells would not hemolyse. In a more hypotonic solution they would reach their critical volume earlier or at the time at which the transmission has the same value as the steady level, $T_s$. Thus the steady state level, $T_s$, obtained in a

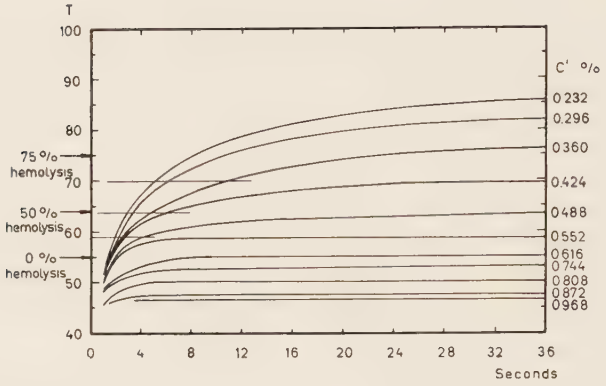



Fig. 18. The rate of swelling and hemolysis of cord blood red cells in different hypotonic NaCl solutions ($C'$). The thin horizontal lines indicate the transmission values for the hemolytic concentrations ($C_h$) 0.424, 0.488 and 0.552 % NaCl. The transmission values corresponding to 0, 50 and 75 % hemolysis are indicated by arrows.

hypotonic solution over a few hours can be used to obtain the time a certain type of cells needs to reach their critical volume in a more hypotonic solution (Fig. 18). Values of the function $f(C'; C_h)$ for the outside concentration ($C'$) used (0.200; 0.232; 0.296; 0.360; and 0.424 % NaCl) and for cells having the hemolytic concentrations ($C_h$) 0.424; 0.488; 0.552; and 0.616 % NaCl were calculated from the equation (6) and are given in Table 4.

The rate of entrance of water into the red cells can also be illustrated by the volume increase in prehemolytic NaCl concentrations. Every degree of hypotonicity corresponds to a definite increase in red cell volume. The relative volume in different outside concentrations can be calculated from equation (1). When $V_o = 1$, $V = C_o/C$, i.e. $V$ expresses a relative volume. The plot of

Table 4

Values of $f(C'; C_h) \times 10^{-3}$ for different outside and hemolytic concentrations of NaCl.

| Outside concentration ($C'$) moles $\times$ cm.$^{-3} \times 10^6$ | Hemolytic concentration ($Ch$) moles $\times$ cm.$^{-3} \times 10^6$ | | | |
|---|---|---|---|---|
| | 72.5 (0.424 %) | 83.5 (0.488 %) | 94.4 (0.552 %) | 105.4 (0.616 %) |
| 34.2 (0.200 %) | 20.8 | 13.7 | 9.5 | 6.5 |
| 39.7 (0.232 %) | 23.1 | 14.9 | 10.1 | 6.9 |
| 50.6 (0.296 %) | 24.6 | 18.1 | 11.7 | 7.8 |
| 61.6 (0.360 %) | 43.0 | 23.1 | 14.3 | 10.4 |
| 72.5 (0.424 %) | — | 33.6 | 18.4 | 11.3 |
| Relative critical volume $\left(Vh = \frac{C_0}{C_h}\right)$ | 2.36 | 2.05 | 1.81 | 1.62 |

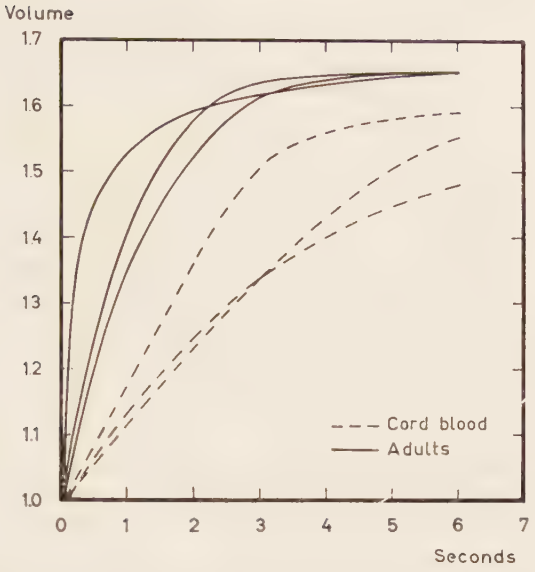



Fig. 19. The rate of relative volume increase of red cells from three adults and three newborn infants (cord blood) in a prehemolytic NaCl solution (0.616 %).

the transmission values at steady state against the corresponding relative volume values gives a straight line (Fig. 16). Such curves can be used as calibration curves for transforming light transmission values to values of relative volumes. Curves of the rate of volume increase can thus be obtained (Fig. 19).

Also from these curves the permeability coefficient can be calculated. The equation (2) can be written

$$P_w = \frac{dV}{dt} \cdot \frac{1}{A\,(C - C')} \cdot \qquad (2\,a)$$

and thus it would be possible to calculate $P_w$, provided that $dV/dt$, $A$, $C$ *and* $C'$ were known. At zero time $C$ and $C'$ as well as $A$ (mean red cell surface area) are known and $dV/dt$, which is the initial slope of the curve of volume increase, can be measured graphically.

## Results

### *Adults*

Unwashed red cells of 3 adults were investigated. Fig. 20 shows the rate of hemolysis at an outside concentration of 0.232 % NaCl and in Fig. 19 the prehemolytic rate of volume increase is given for an outside concentration of 0.616 % NaCl.

The mean value of $P_w A/V_o$ was found to be $35.8 \times 10^6$ for cells with hemolytic concentrations ($C_h$) of 0.552 and 0.488 % NaCl (Adults (1) Fig. 21, Table

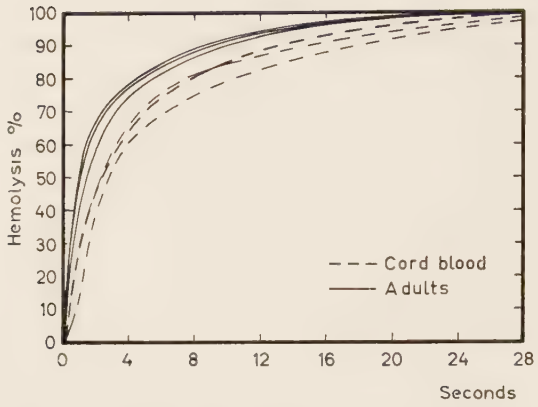



Fig. 20. The rate of hemolysis of the red cells from three adults and three newborn infants (cord blood) in a 0.232 % NaCl solution.

5), and $13.8 \times 10^6$ for cells with a hemolytic concentration of 0.424 % NaCl (Adults (2)). The value of $P_w A / V_o$ for washed red cells with a hemolytic concentration of 0.488 % NaCl was found to be the same as for unwashed cells.

The mean volume of adult red cells is 87 $\mu^3$, the effective osmotic volume of such a cell is about $0.70 \times 87 = 61$ $\mu^3$ and the surface area is 163 $\mu^2$. Using these figures the permeability coefficient, $P_w$, was found to be 1339 for adult red cells with hemolytic concentrations of 0.552 and 0.488 % NaCl. For adult cells with a hemolytic concentration of 0.424 % NaCl $P_w$ was 510. Calculated from the rate of swelling in prehemolytic NaCl solutions a $P_w$ of about 1000 was found (Adult (3)). The corresponding values of $P$ were $22.1 \times 10^{-3}$, $8.4 \times 10^{-3}$, and $16.5 \times 10^{-3}$ cm sec.$^{-1}$ (Table 5).

*Fetuses*

In one fetus (16 cm long, 16 weeks duration of pregnancy) the mean value of $P_w A / V_o$ was found to be $14.5 \times 10^6$ for red cells with hemolytic concentrations of 0.616 and 0.424 % NaCl (Fig. 21, Table 5). Since the dimension of the red cells are not very well known, the permeability coefficient has not been considered possible to calculate.

*Cord blood*

Red cells from the cord blood of 5 infants were investigated. Fig. 20 shows the rate of hemolysis at an outside concentration of 0.232 % NaCl and the prehemolytic rate of volume increase is given in Fig. 19. They show that the fetal red cells swell and hemolyse slower than the adult red cells.

The mean value of $P_w A / V_o$ was found to be $17.9 \times 10^6$ for cells with hemolytic concentrations of 0.552 and 0.488 % NaCl (Cord blood (1), Fig. 21, Table 5). In two of the infants the mean value of $P_w A / V_o$ was found to be

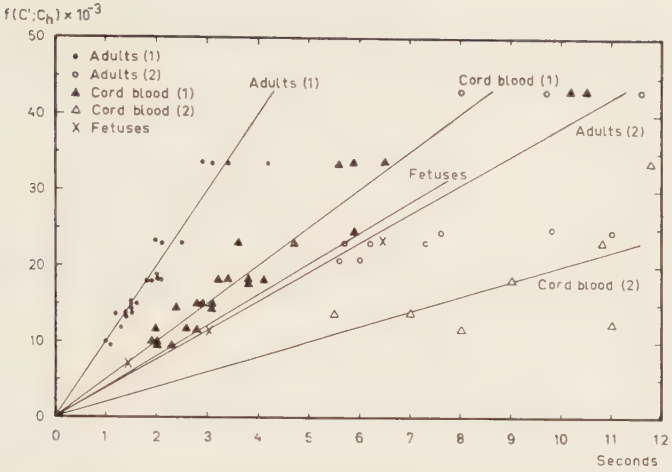



Fig. 21. $f(C'; C_h)$ of different red cells plotted against time of hemolysis. The slope $(P_w A / V_0)$ for Adults (1) = $35.8 \times 10^6$, for Adults (2) = $13.8 \times 10^6$, for Cord blood (1) = $17.9 \times 10^6$, for Cord blood (2) = $7.2 \times 10^6$, and for Fetuses = $14.5 \times 10^6$.

$7.2 \times 10^6$ for hemolytic concentrations of 0.488 and 0.424 % NaCl (Cord blood (2)).

The respective permeability coefficients ($P_w$) were found to be 708 and 283 assuming that the mean red cell volume was 106 $\mu^3$, the osmotically

TABLE 5

The permeability of red cells to water.

The experimental values are calculated from the rate of hemolysis of red cells with the higher hemolytic concentrations (1), from the rate of hemolysis of red cells with the lower hemolytic concentrations (2), and from the rate of volume increase of red cells in prehemolytic NaCl solutions (3).

| | | $P_w A/V_0$ cm$^3$ hour$^{-1}$ moles$^{-1}$ | $P_w$ cm$^4$ hour$^{-1}$ moles$^{-1}$ | $P$ cm sec.$^{-1}$ |
|---|---|---|---|---|
| Adults | (1) | $35.8 \times 10^6$ | 1339 | $22.1 \times 10^{-3}$ |
| Adults | (2) | $13.8 \times 10^6$ | 510 | $8.4 \times 10^{-3}$ |
| Adults | (3) | — | 1000 | $16.5 \times 10^{-3}$ |
| Fetus | | $14.5 \times 10^6$ | — | — |
| Cord blood | (1) | $17.9 \times 10^6$ | 708 | $11.7 \times 10^{-3}$ |
| Cord blood | (2) | $7.2 \times 10^6$ | 283 | $4.7 \times 10^{-3}$ |
| Cord blood | (3) | — | 400 | $6.6 \times 10^{-3}$ |
| Full-term infant aged 29 hours | (1) | $19.2 \times 10^6$ | — | — |
| | (2) | $11.0 \times 10^6$ | — | — |
| Premature infant aged 28 hours | (1) | $20.6 \times 10^6$ | — | — |
| | (2) | $11.0 \times 10^6$ | — | — |
| Adults (according to JACOBS) | | — | 400 | $6.6 \times 10^{-3}$ |

effective volume $0.70 \times 106 = 74\ \mu^3$, and the surface area 187 $\mu^2$. Calculated from the rate of swelling in prehemolytic NaCl solutions a $P_w$ of about 400 was found (cord blood (3)). The corresponding values of $P$ were $11.7 \times 10^{-3}$, $4.7 \times 10^{-3}$, $6.6 \times 10^{-3}$ cm sec.$^{-1}$ (Table 5).

*Newborn infants*

In one full-term infant aged 29 hours the mean value of $P_w A / V_o$ was found to be $19.2 \times 10^6$ for cells with hemolytic concentrations of 0.552 and 0.488 % NaCl and $11.0 \times 10^6$ for cells with a hemolytic concentration of 0.424 % NaCl (Table 5).

In one infant born 6 weeks prematurely and aged 28 hours the mean value of $P_w A / V_o$ was found to be $20.6 \times 10^6$ for cells with a hemolytic concentrations of 0.488 % NaCl and $11.0 \times 10^6$ for cells with a hemolytic concentration of 0.424 % NaCl (Table 5).

## Discussion

*Water permeation with regard to the structure of the red cell membrane*

For adult red cells the permeability coefficient ($P$) for water was $22 \times 10^{-3}$ cm sec.$^{-1}$. It would be of interest to see if the permeation of water through the red cell membrane can be ascribed to a process of free diffusion. If this were the case the permeability coefficient would be equal to the free diffusion coefficient ($D$) divided by the thickness ($\delta$) of the red cell membrane.

The above permeability coefficient was calculated assuming that the whole cell surface was available for the permeation of water. It is, however, considered impossible that the permeation occurs all over the area of the cell surface. Lipid insoluble substances are generally assumed to pass the membrane through water-filled pores. Parpart and Ballentine (1952) as well as Hillier and Hoffman (1953) calculated the number and size of the pores. They gave a pore number of about $10^4$–$10^5$ per red cell and a pore diameter of about 35 Å. According to the present knowledge of the structure of the membrane the pores do not contain only water but also lipo-protein molecules. The area available for permeation of the water molecules must thus be less than corresponding to a pore diameter of 35 Å. Because sucrose, which has a molecular diameter of about 9 Å, does not pass the red cell membrane, a diameter of 10 Å for water permeation channels is assumed to be a reasonable approximation. The pore area is thus about 80 Å$^2$. If the total number of pores per cell is assumed to be $3 \cdot 10^5$ (the highest value given by Parpart and Ballentine 1952), the total cross-sectional area available for water permeation will be $24 \times 10^6$ Å$^2$, which is 0.15 % of the total area of the red cell ($16 \times 10^9$ Å$^2$). Accordingly, the permeability coefficient

will be 666 times greater than the value given above, i.e. 13 cm sec. $^{-1}$. Since the self-diffusion coefficient for water at 20° C is about $2 \times 10^{-5}$ cm$^2$ sec. $^{-1}$ (Wang, Robinson and Edelman 1953), and the thickness of the red cell membrane is assumed to be about 75 Å $= 0.75 \cdot 10^{-6}$ cm, $D\ \delta$ will be 27 cm sec. $^{-1}$. As to the thickness of the membrane some further comments will be made. The value of 75 Å is in itself uncertain. Owing to the presence of "unstirred layers" on each side of the membrane, it is possible that the "effective" membrane thickness is greater (Teorell 1937). Especially on the inside, where no "stirring" exists, this "unstirred layer" must be comparatively great. The value of 75 Å, however, may be used as an approximation consistent with the present opinion of the membrane structure. Below this value, approximately, Fick's law is not applicable, because the concept of concentration gradients loses its usual meaning when the membrane thickness becomes of the order of distances between the solute molecules (Planck, cit. from Teorell 1937).

These approximate calculations thus give a *permeability for water that is restricted by a factor of about two in comparison with free water diffusion* (13 compared with 27 cm sec. $^{-1}$). Such a small restriction seems quite plausible because the pore size is relatively large (10 Å diameter) compared with the size of the water molecule, which has a diameter of about 2–3 Å.

For larger molecules the relative restriction will be successively increased until the molecules reach the pore size and no permeation at all is possible, as is the case with sucrose. The relation between the relative restriction and the ratio "pore size molecular size" is probably not a straight line. The relative restriction must be assumed to increase much more rapidly than the ratio "pore size/molecular size" decreases (cf. the discussion of Pappenheimer 1953).

For the lipid insoluble substances urea and mannitol the diffusion coefficients in aqueous media and the permeability to red cells are known. If the figures given by Jacobs (1952) are used, the corresponding calculations can be made also for these substances. The permeability of urea, which has a molecular diameter of about 3–4 Å, is restricted about 120 times in comparison with its free diffusion in water. Mannitol, which has an even larger molecular diameter, is restricted about 6000 times.

The pore size, however, is certainly varying, and it is quite possible that water, urea and mannitol all pass practically unrestricted through some large pores, while water is the only substance that passes also through the smallest ones. With increasing size of the molecules an entirely regular increase in relative restriction is thus not to be expected.

Though a calculation of this type must always be very approximate, it is nevertheless very interesting to see that *the present opinion of the red cell*

*membrane structure seems to be in good accordance with the information obtained by permeability studies on human red cells.*

*Variation of the permeability coefficient of red cells of the same blood*

JACOBS found the permeability coefficient of *adult red cells* for water ($P_w$) to be about 400. If this figure is converted to cm sec.$^{-1}$, a permeability coefficient ($P$) of $6.6 \times 10^{-3}$ is obtained. This value is about three times lower than the value obtained in the present investigation.

In this investigation the permeability coefficient varied with the degree of hemolysis, a high degree of hemolysis corresponding to a low permeability coefficient and vice versa. At 75 % hemolysis JACOBS got a permeability coefficient of $6.6 \times 10^{-3}$, while the present investigation gave a $P$ of $8.4 \times 10^{-3}$ at about 65 % hemolysis and a $P$ of $22.1 \times 10^{-3}$ at degrees of hemolysis lower than about 50 % (Fig. 18).

It should also be stressed that the higher $P$ values are associated not only with the lower degrees of hemolysis but naturally also with the higher critical concentrations ($C_h$) and the corresponding lower critical volumes ($V_h$).

Looking for an explanation of the varying $P_w$ or $P$ values the variables influencing the value of $P$ may be obtained from equation (6). $V_o$ and $C_o$ are constant in the same blood. $C'$ is not changed during the experiments, because the total volume of the red cells is small in comparison with the volume of the NaCl solution. The time, $t_h$, is established experimentally.

The two remaining factors, $A$ and $C_h$, demand a more detailed discussion.

The surface area, $A$, is not constant throughout an experiment. During the disk-sphere transformation the area is supposed to behave in the following way. Until the red cell becomes a sphere the surface area increases only slightly. Then it suddenly decreases and thereafter a continuous increase takes place until hemolysis occurs and the area has become about the same as that of the initial red cell (PONDER 1948a, p. 82). During the later stages of this process derangements of the membrane structure probably also occur (LATTA 1952). Such derangements may alter the permeability properties of the cell membrane.

The equation (6) is based on the assumption that the value of $C_h$ corresponds to the critical volume, $V_h$. According to the equation (1) $V_h$ is equal to $C_o/C_h$ if $V_o = 1$. At lower $C_h$ values, however, this relation is not true, because $V_h$ becomes greater than the observed maximal critical volume of a red cell (about 1.7; Table 4). At the higher $V_h$ values the relation $C_o/C_h$ thus does not indicate the critical volume of the red cells, but is only a calculation product.

The equation (6) was also based on the assumption that the red cell is a perfect osmometer without selective permeabilities for ions like K and Na.

Because a red cell becomes cation permeable *before* it hemolyses (PONDER 1948a), equation (6) is valid only during the first stages of the process of swelling. Thereafter the K of the cell is exchanged for Na. These two ions, however, do not have the same rate of transfer. The K ions have a higher mobility in water than the Na ions. TEORELL (1952) calculated the diffusion coefficient ratio Na : K to be about 0.8. The rate of transfer however, is also influenced by the concentration gradients of the ions. In whole blood the concentration difference between cell K and plasma K is $81 - 5 = 76$ meq./lit. (PONDER 1948 a, p. 121). Between cell Na and plasma Na the difference is $18 - 135 = -117$ meq./lit. The net rate of transfer is proportional to the diffusion coefficient and to the concentration gradient of the ion. If the red cells of whole blood become cation permeable "the ratio the initial rate of transfer of Na/the initial rate of transfer of K" is $0.8 \cdot \frac{117}{76} = > 1$. The Na ions thus change more rapidly and the result is an increased total base content of the cells, and concequently also a water uptake and a swelling of the cells (cf. MAIZELS 1949). Not until the ratio "Na concentration gradient / K concentration gradient" is 1.25, is the net rate of transfer of both ions equal (i.e. $0.8 \times 1.25 = 1.0$). The present discussion, however, concerns hypotonic NaCl solutions where the initial concentration gradients are not the same as in whole blood. The K concentration gradient is about 81 meq./lit., because there are no K ions in the NaCl solutions, and the Na concentration gradient is varying depending on the concentration of the outside NaCl solution. In all NaCl concentrations, however, in which hemolysis occurs, i.e. below about 95 meq./lit., there is a greater initial concentration gradient for K (81 meq./lit.) than for Na ($18 - 95 = -77$ meq./lit.). Then $0.8 \times$ Na concentration gradient/K concentration gradient is always $< 1.0$ and the K loss is thus more rapid than the Na uptake. Because isotonicity inside and outside the red cell is always maintained, the amount of water leaving the red cell with the K ions is larger than the amount of water entering the cell with the Na ions. The prolytic K loss thus causes a decreased total water uptake and gives a lowered permeability to water. This apparent decrease in permeability can be expected to be most marked in the higher degrees of hemolysis where a comparatively great number of the red cells are prolytic spherocytes.

*In the lower hemolytic range, i.e. at the lower $V_h$ values P is remarkably constant and there is a good fit to the equation* (6) (Fig. 21, Adults (1)). The poor fit to the equation (6) in the higher hemolytic range (Fig. 21, Adults (2)) may well depend on the changes in surface area and in the membrane structure and on the prolytic K loss during the experiment. The fact that the relation $C_o/C_h$ does not indicate the real $V_h$ at low $C_h$ values certainly also plays a role. *The higher P value thus seems to be the more reliable one.*

The $P$ values obtained from the rate of swelling of the red cells in the prehemolytic NaCl solutions (Adults (3), Table 5) are intermediate to the above discussed values. Because the swelling of the red cells can be determined directly in these experiments, the $P$ values obtained from them are very satisfactory from a theoretical point of view. The determinations, however, are subject to relatively great errors, because the volume increase is measured from rather small changes in transmission. In any case it is interesting to note the good agreement between the different methods, by which the permeability coefficients have been calculated.

The permeability coefficient of *cord blood red cells* showed quite a similar decrease with increasing hemolysis (Fig. 21, Cord blood (1) and Cord blood (2)). In one case the lower $P$ value was observed already at a hemolytic concentration of 0.488 % NaCl.

The *cells of the only fetus* investigated, on the contrary, showed a constant $P$ at hemolytic concentrations between 0.616 and 0.424 % NaCl. This is in good agreement with the findings of WIDDAS (1951) in sheep fetal and maternal red cells.

*Difference in permeability coefficients of adult and fetal red cells*

Before ascribing the difference in water permeability of adult and fetal red cells to membrane differences some further factors have to be regarded.

Equation (6) shows that the permeability coefficients is influenced by changes in the ratio "surface area/osmotically effective volume" ($A/V_o$). In adult red cells the osmotically effective volume can be estimated to be about 70 % of the total volume (PONDER 1948a). It is not known whether the osmotically effective volume of adult and fetal red cells constitutes the same percentage of the total cell volume. There is, however, no reason to believe that any great differences exist and it may be permissible to use the normal mean values of surface areas and volumes when the ratio is calculated. The difference between the ratio "surface area/volume" of adult and fetal red cells is about 7 %, the surface area of the fetal cells being the relatively larger one. $P$ of adult red cells, however, is about 50 % greater than $P$ of fetal red cells and the difference in $P$ can not be explained by the small difference in the ratio "surface area/volume" of adult and fetal red cells.

If there were great differences in the critical volume of adult and fetal red cells, different permeability coefficients were to be expected. Since both adult and fetal red cells have been shown to be perfect osmometers under the actual conditions, the relation $V_h/V_o$ is the same for both types of cells and the difference in $P$ cannot be connected with this factor.

*It thus seems probable that the difference in permeability of adult and fetal red cells to water depends on differences in their membrane properties.*

CHAPTER V

# The Mechanical Resistance of the Red Cells of Fetuses, Children and Adults

## Earlier Investigations

The red cells are presumably subject to a considerable "wear and tear" in the circulation. Consequently, the mechanical stress in vivo has been supposed to play a role in the normal red cell destruction. There is, however, no definite experimental evidence in favour of this supposition

Shen, Castle and Fleming (1944) introduced a method for determining the mechanical resistance of red cells in vitro. In normal adults they found only small variations. In congenital hemolytic anemia, pernicious anemia, and sickle cell anemia as well as in the presence of iso-agglutinins and cold agglutinins they demonstrated a lowered mechanical resistance of the red cells. The factors thought to be responsible for a decreased mechanical resistance were increased spheroidicity, increased cohesion between the red cells, and decreased strength of the red cell membrane. The results concerning hemolytic anemias have been largely confirmed by Young (1947), Matthes (1950), and Young, Izzo and Platzer (1951).

Using radioactive iron on dogs, Stewart *et al.* (1950) showed that the mechanical resistance of young red cells was higher than that of the general red cell population. The oldest cells, on the other hand, had a lower mechanical resistance than the general population.

A decreased mechanical resistance thus often seems to reflect a shortened in vivo survival of the red cells. "Inherent defects of the red cell structure, as well as injury to the red cell surface ultrastructure seem to be the principal causes of increased mechanical fragility" (Ponder 1948).

The mechanical resistance of the red cells of infants and children was investigated by Goldbloom *et al.* (1953). They found a decreased mechanical resistance in infants during the first 6 days of life in comparison with adults. No determinations were made in infants aged 6 days to 6 months. In children aged 6 months to 13 years the mechanical resistance was the same as in the adults. A possible relationship between a decreased resistance and an increased red cell destruction in vivo during the

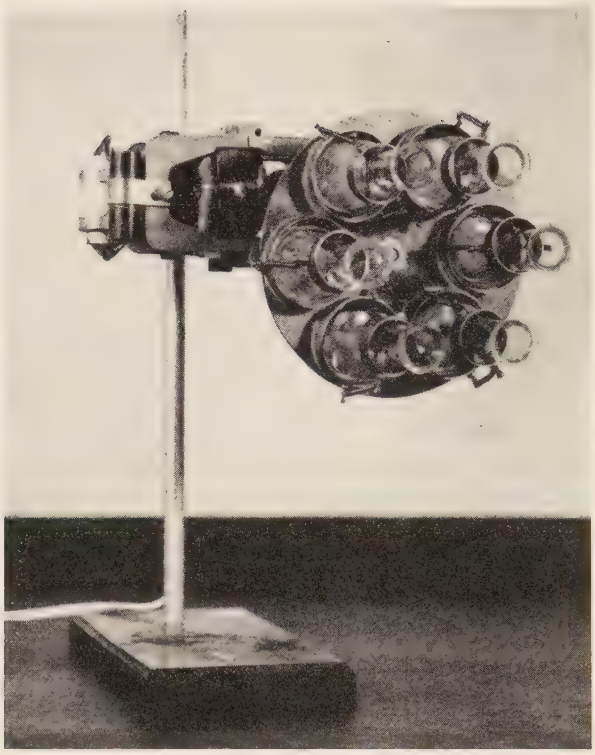


Fig. 22. Photograph of the mechanical rotator.

neonatal period was suggested. Another support of such a relation was given by Hsia, Goldbloom and Gellis (1954), who found that the degree of physiological jaundice in newborn infants varied with the red cell susceptibility to mechanical trauma in vitro. When these papers of Goldbloom *et al.* were published the present investigation on the mechanical resistance of the red cells during infancy and childhood was practically completed.

## Methods

The *blood samples* were obtained as above (p. 18).

The red cells were subjected to *mechanical trauma* in the rotator shown in Fig. 22. The distance from the center of the flasks to the center of the rotating wheel was 5.5 cm. The hematocrit was adjusted to 35 % with its own plasma. Heparinized whole blood was used and a sample of 0.5 ml was rotated for 60 minutes at 30 r.p.m. in a 50 ml Erlenmeyer flask (Pyrex) containing 10 spherical glass beads with a diameter of 3 mm and weighing 0.052 g each. The test was performed at room temperature varying between 18 and 22° C.

The *degree of hemolysis* of the rotated sample was estimated using the method described above (p. 19). The degree of hemolysis of an unrotated sample and of a

sample completely hemolysed in distilled water was estimated in order to obtain a calibration curve. *The red cell susceptibility to mechanical trauma* is usually expressed as the percentage hemoglobin liberated during the rotation. Because the term resistance has been used in this paper (instead of fragility), the mechanical resistance was expressed as the percentage of undestroyed red cells, i.e. 100 minus the percentage of hemoglobin liberated during the rotation.

The *error* was ± 4 % when calculated from 100 duplicate determinations.

During the experiments on the mechanical resistance some *methodological observations* have been made, which stress the importance of a detailed standardization of the method. Even small differences in the hematocrit give differences in the degree of hemolysis; therefore the hematocrit of the tested blood sample has to be adjusted to a certain value. The degree of damage is easily changed by changing the number of glass beads or the revolution rate or the total time of rotation. In one case an increase in revolution rate from 30 to 60 r.p.m. gave a lowering of the number of resistant cells from 95 % to 91 %. The influence of the time of rotation is shown in Fig. 23. The number of glass beads seems to influence the mechanical resistance less than the revolution rate or the time of rotation. An increase of the number of glass beads from 10 to 20 gave no definite decrease in the number of resistant cells. Instead of whole blood washed red cells may be used, but the results are then much more inconstant. These scattered observations indicate that a closer study of the different factors influencing the mechanical resistance test might be valuable.

The *osmotic resistance* was tested as above (p. 19).

The *reticulocytes* were counted as above (p. 19).

The *red cell diameter distribution* was obtained from 1000 × magnified microphotographs of dried blood films.

Concerning *material* see p. 24.

## Results

The results of the determinations of the mechanical resistance are shown in Table 6.

### *Full-term infants*

The red cells at birth and during the first two to three months of life were less resistant to mechanical trauma than adult red cells. At birth and during the first two days of life the mechanical resistance was lower than after the first week of life. After the third month the mechanical resistance was practically the same as in the adults.

For the purpose of investigating whether the decreased mechanical resistance of the red cells of the cord blood was a characteristic of a few cells only, three cord blood samples and five adult blood samples were subjected to an increased mechanical shaking during a prolonged time (20 glass beads, 80 r.p.m., 5 hours). The result is shown in Fig. 23. Obviously a relatively great number of the late fetal red cells were more susceptible to mechanical trauma than adult red cells.

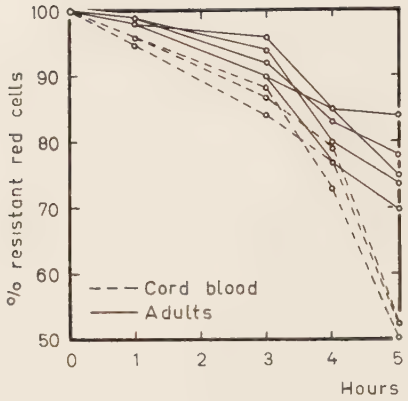



Fig. 23. Relation between the mechanical resistance of red cells and the time of shaking.

TABLE 6

The mechanical resistance of the red cells of fetuses, children and adults.

| Age group | No. of cases | % resistant cells | | |
|---|---|---|---|---|
| | | Mean | Standard error of the mean | Range |
| Fetuses | 5 | 90.7 | ±3.4 | 79.0– 97.5 |
| Cord blood | 29 | 95.8 | ±0.46 | 89.0– 99.0 |
| 12–48 hours | 17 | 94.6 | ±0.53 | 92.0– 99.0 |
| 6–10 days | 10 | 96.4 | ±0.20 | 96.0– 97.5 |
| 15–45 days | 10 | 95.6 | ±0.43 | 92.5– 97.0 |
| 46–75 days | 10 | 95.6 | ±0.59 | 92.5– 98.0 |
| 76–105 days | 14 | 96.8 | ±0.31 | 97.0– 99.0 |
| 4–6 months | 10 | 96.6 | ±0.42 | 96.0– 98.0 |
| 10–12 months | 10 | 98.1 | ±0.27 | 97.0– 99.0 |
| 2–5 years | 10 | 98.4 | ±0.25 | 97.0– 99.5 |
| 7–11 years | 15 | 98.2 | ±0.20 | 97.0–100.0 |
| Adults | 38 | 98.0 | ±0.34 | 95.0–100.0 |

*t* test on the differences of the mean of the mechanical resistance.

| Compared age groups | Difference | Probability of identity |
|---|---|---|
| Cord blood *vs.* fetuses | 5.1 | $0.10 < P < 0.50$ |
| Cord blood *vs.* 12–48 hours | 1.2 | $0.05 < P < 0.10$ |
| Cord blood *vs.* 4–6 months | −0.8 | $0.10 < P < 0.50$ |
| Adults *vs.* cord blood | 2.2 | $P < 0.001$ |
| Adults *vs.* 12–48 hours | 3.4 | $P < 0.001$ |
| Adults *vs.* 15–75 days | 2.4 | $P < 0.001$ |
| Adults *vs.* 4–6 months | 1.4 | $0.01 < P < 0.02$ |

*Immature infants*

In three infants born at least 4 weeks prematurely, the mechanical resistance of the red cells of the cord blood was 91, 95 and 96 %, which values are within the limits of the full-term infants. In four infants born at least 5 weeks prematurely and aged from 9 to 32 days the mechanical resistance was 88, 87, 98, and 92 %.

*Fetuses*

In the few young fetuses investigated the mechanical resistance seemed to be lower than in the cord blood of the full-term infants. The difference, however, was not significant.

*The mechanical resistance of reticulocytes*

The mechanical resistance of reticulocytes was investigated by counting the number of reticulocytes before and after a prolonged mechanical resistance test (80 glass beads, 80 r.p.m., 3 hours). The fresh, untreated blood contained 1.6 % reticulocytes. After the shaking 32 % of the red cells were destroyed. Of the undestroyed red cells 6 % were reticulocytes. An untreated blood sample showed 2 % reticulocytes after three hours at room temperature. If it is assumed that no reticulocytes had been destroyed during the mechanical resistance test a value of 2.3 % reticulocytes would be expected after the shaking. The observed value of 6 % is much higher and thus the number of reticulocytes apparently increased during the test.

*The relation between the red cell shape, the osmotic resistance and the mechanical resistance*

Before and after the shaking of two cord blood samples and one adult blood sample, the osmotic resistance was tested, the red cells were photographed and the cell diameters were measured. A shaking that destroyed about 20 % of the red cells gave an increased osmotic resistance (Fig. 24a). The microphotograph showed that about 15 % of the undestroyed cells were more or less crenated, while the others looked rather normal (Fig. 25). The cell diameter distribution was practically identical before and after the shaking, only the smallest cells (spherocytes?) had quite disappeared (Fig. 26).

A more violent shaking that destroyed 30 to 50 % of the red cells gave irregular osmotic resistance curves (Fig. 24b). One part of the cells had be-

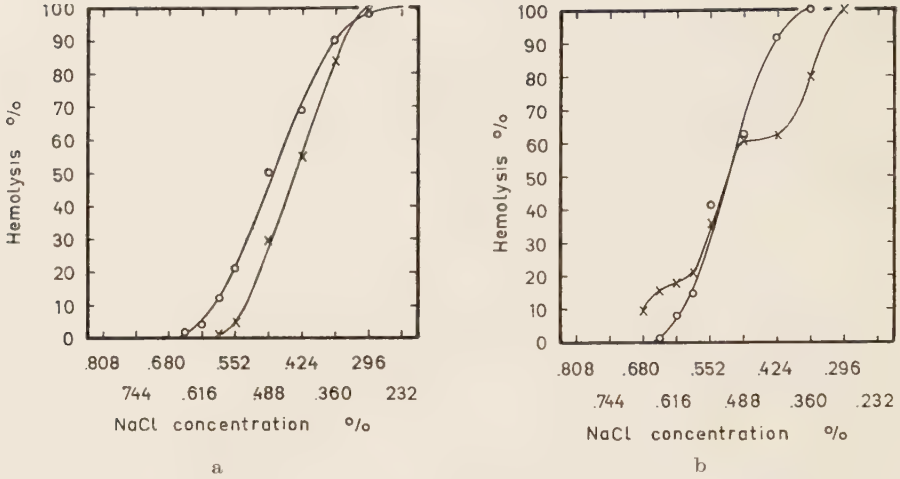



Fig. 24. Degree of hemolysis of cord blood red cells at different NaCl concentrations before (○) and after (×) about 20 % (Fig. 24a) and 50 % (Fig. 24b) of the red cells had been destroyed by shaking.

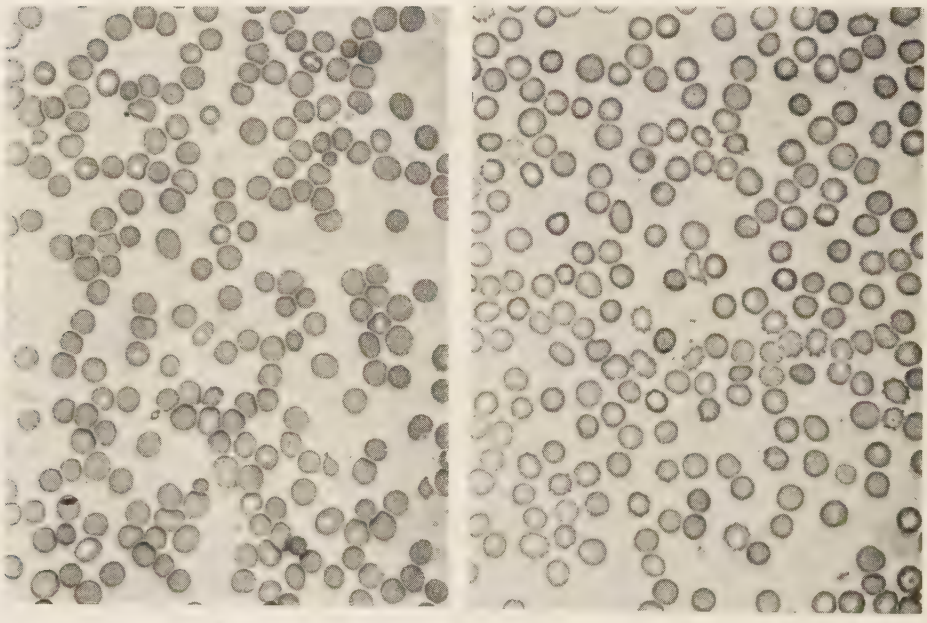



Fig. 25. Microphotographs of the red cells before (Fig. 25a) and after (Fig. 25b) about 20 % of the red cells had been destroyed by shaking.

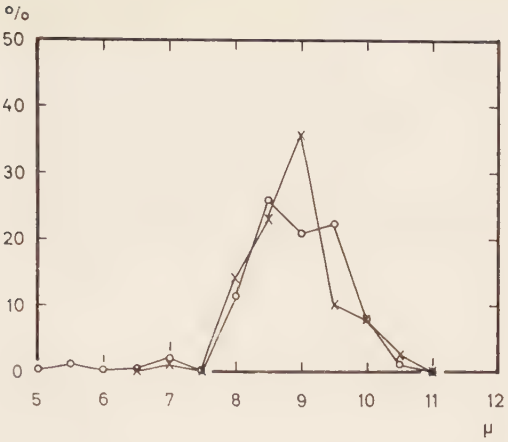



Fig. 26. The red cell diameter distribution before (○) and after (×) about 20 % of the red cells had been destroyed by shaking.

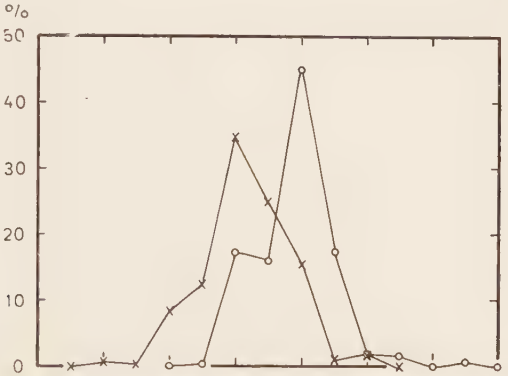
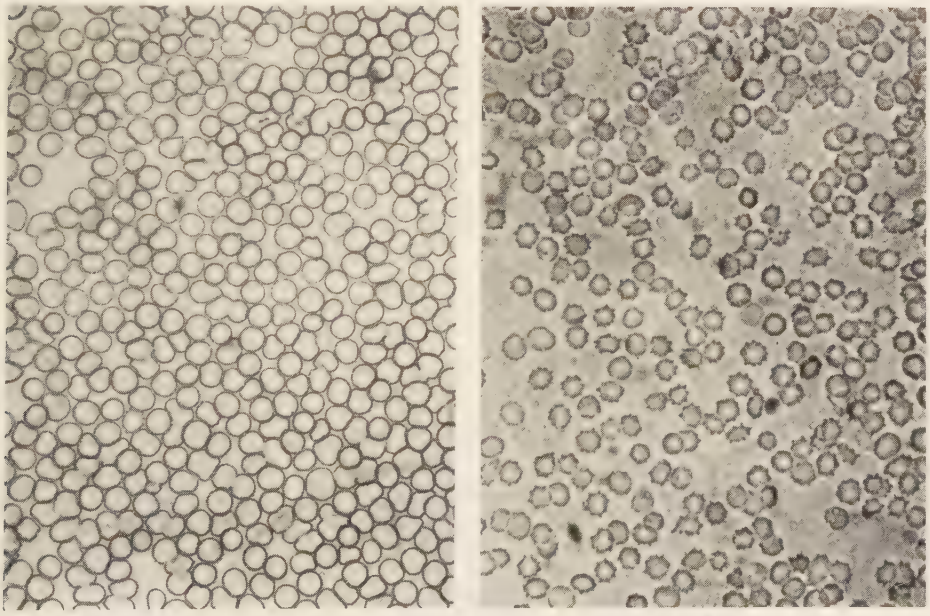



Fig. 28. The red cell diameter distribution before (○) and after (×) about 50 % of the red cells had been destroyed by shaking.



a b

Fig. 27. Microphotographs of the red cells before (Fig. 27a) and after (Fig. 27b) about 50 % of the red cells had been destroyed by shaking.

come more resistant and another part less resistant than before the shaking. The microphotograph showed that all undestroyed red cells had become crenated and distorted (Fig. 27). The distribution of the red cell diameters had changed markedly, the smallest cells had increased in number and the mean cell diameter had decreased from 8.9 to 8.2 μ (Fig. 28).

## Discussion

It is evident that *the majority of the fetal red cells are more susceptible to mechanical trauma* in vitro than adult red cells. The present investigation cannot prove that this increased mechanical in vitro destruction of the red cells in the neonatal period indicates an increased in vivo destruction. Goldbloom *et al.* (1953 and 1954), suggested that there exist such a relationship, which also is in accordance with Mollison's (1951) finding that some cord blood red cells had a shortened post-transfusion survival.

Goldbloom *et al.* did not investigate infants between 6 days and 6 months, but concluded from their values at the end of the first week of life that the mechanical resistance was normal after this time. According to the present investigation it seems probable that the mechanical resistance is decreased also after the first week of life. Normal adult values were not obtained until the second to third month of life. It may be worth mentioning that the increase in mechanical resistance on the whole parallels the disappearance of the large fetal red cells (Fig. 2).

The figures obtained for the mechanical resistance of adult red cells agree very well with those given by other investigators. The small differences might be ascribed to methodological divergences.

The finding that the reticulocytes belong to the mechanically most resistant cells is in good accordance with the investigations of Stewart *et al.* (1950), who showed that young red cells were more resistant to mechanical stress than old cells. The absolute increase in the number of reticulocytes after the mechanical resistance test is rather astonishing. A possible explanation may be that more reticulocytes have become stainable when the cells swell and crenate during the test. The phenomenon seems to be a parallel to the increased number of reticulocytes found in prehemolytic hypotonic NaCl solutions (cf. p. 35), in which the red cells also are swollen and crenated.

The relation between the osmotic and the mechanical resistance has been discussed by different authors, but no regular relationship has been found (Young 1947; Stewart *et al.* 1950). *With regard to normal cells*, however, the present investigation has shown that *the mechanically most resistant red cells also seem to be the osmotically most resistant ones.* In several pathological cases,

however, with signs of decreased in vivo survival, discrepancies between the mechanical and the osmotic resistance have been observed. As a rule the mechanical resistance in these cases was decreased, while the osmotic resistance was normal. It has equally been shown that, before bursting, the red cells subjected to shaking became crenated and probably also more spherocytic.

CHAPTER VI

# The Storage Behaviour of the Fetal Red Cells in Comparison with the Adult Red Cells

## Earlier Investigations

The behaviour of red cells during storage has been investigated thoroughly in connection with blood preservation studies. (For literature see RAPOPORT 1947 and GIBSON 1953).

Differences in the behaviour of stored red cells depend on the preservative solution, on the temperature, and on the red cell properties. On the whole, however, the storage changes follow a common scheme; the rate of the changes is different under different conditions only. The changes of the stored red cells have been attributed to a failure of the phosphorylation of glucose (DENSTEDT 1953). The red cell content of glucose, adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), and of diphosphoglycerate decreases, while the content of inorganic phosphate, lactic acid, and pyruvic acid increases. Meanwhile the pH of the cells decreases. The normal in vivo concentration gradients of potassium and sodium depend on the metabolic activity of the red cells. When the red cell metabolism fails, potassium diffuses out of the cell and sodium enters it according to usual diffusion laws. Because the rate of change of sodium is greater than that of potassium, the total base content of the red cells increases during storage (cf. the discussion, p. 55). Accordingly, the red cells swell and the osmotic resistance decreases.

During storage at $-4°$C the glycolytic metabolism of the red cells is very much decreased and the cation control is lost practically at once. During storage at $-37°$ C the glycolytic processes are thought to proceed at the in vivo rate for a certain time. As soon as the cells are deprived of most of their glucose and energy-rich phosphates, the metabolism, however, fails and the cation control is lost. On account of the higher temperature the cation equilibration proceeds faster at $+37°$ C than at $+4°$ C, once it has started.

Attempts have been made to correlate the red cell survival in vivo with the physical and chemical changes occurring during the storage in vitro. At present, however, there is no in vitro test that parallels the in vivo survival. MOLLISON and YOUNG (1941 and 1942) denied every correlation

between in vitro tests on preserved red cells and the survival of these cells in vivo after transfusion. Some authors on the other hand are of the opinion that definite correlations exist between in vitro changes and in vivo survival, even if the in vitro tests do not give a finite prediction of the in vivo behaviour. RAPOPORT (1947) found that in vitro and in vivo results correspond rather well, and he recommended in vitro tests as a preliminary procedure for the screening of preservative solutions and for the investigation of the mechanisms of the deteriorative processes of the red cells. According to GIBSON (1953) "the effect upon in vivo survival of the abnormality in behaviour of the red cell occurring in the preservative solution was very striking". PENNELL (1953) stated that the osmotic resistance and the glycolytic activity of the red cells give a valuable laboratory forecast of the probable in vivo survival of the red cells. DENSTEDT (1953) claimed that there appears to be a fair correlation between the viability of the cells and the ATP. GABRIO and FINCH (1954), however, showed that the changes during in vitro storage in acid-citrate-dextrose (ACD) bear no relationship to in vivo senescence of the erythrocyte during the first 24 hours following transfusion. The typical storage alterations were not found during the aging of red cells in vivo. Judging from experiments on dogs concerning the in vivo survival 6 and 12 hours after transfusion, they concluded that young red cells are more susceptible to storage damage than older cells. GABRIO, STEVENS and FINCH (1954) were also able to demonstrate that some extra-erythrocyte factors (citrate, plasma age, leucocyte and reticulocyte concentration) have little effect on the red cells during storage. The same authors also showed that the storage damage of the majority of the red cells was rapidly reversed once the red cells were introduced into active circulation.

In the present investigation the changes of stored cord blood and adult blood have been compared in order to reveal possible differences between the fetal and the adult red cells. The degree of spontaneous hemolysis, the decrease in mechanical and osmotic resistance, and the K loss of the cells after different times of storage at + 4° C and + 37° C have been estimated.

## Methods

Heparinized *cord whole blood* and *adult whole blood* was collected under sterile conditions. Three to eight samples of about 10 ml each were then stored in stoppered, sterilized, 50 ml Erlenmeyer flasks (Pyrex) at + 4° C and/or + 37° C. In some experiments *washed red cells* suspended in sterile Salidex$^{R}$ (Pharmacia Ltd, Uppsala)[1] were used.

[1] Salidex$^{R}$ is an electrolyte solution for infusion containing 9 g NaCl, 0.3 g KCl, 0.2 g $CaCl_2$, 0.05 g $MgCl_2$, 0.6 g 20 % Na lactatis, 1 g Sacchar. invert. monhydr., and aqua steril. ad 1000 ml (pH varying between 5.5 and 7.0).

The changes were measured after different times of storage. The content of one flask was used every time.

The degree of *spontaneous hemolysis* was measured photometrically in 0.968 % NaCl, following the method used in the osmotic resistance test (p. 19).

The *mechanical resistance* was measured as above (p. 58). The change in mechanical resistance was expressed as the difference between the value at zero time and the values at the different times of storage.

The *osmotic resistance* was measured as above (p. 19). As a rule the osmotic resistance curves were shifted to the left during the storage. Because the same modul was used in every diagram, the area enclosed between two osmotic resistance curves of the same blood at different times of storage seemed to be a fairly good measure of the total shift of the osmotic resistance. These "*shift areas*" (in $cm^2$) have been used when the changes in the osmotic resistance of different blood samples were compared. Coradi's Compensations-Planimeter was used for measuring the areas.

The *hematocrit* was measured as above (p. 18).

The *red cell diameter distribution* was obtained from 1000 × magnified microphotographs of dried blood films.

*Potassium determinations* were performed with a Perkin-Elmer flame photometer (model 52 A with internal Li standard). The K concentration of the red cells was calculated from the determined K values of hemolysed whole blood samples and of the plasma of the same blood. All K concentrations were corrected to the original cell volume. No correction was made for the trapped plasma, which can be estimated to be about 1 % with the hematocrit method used (Enghoff, 1954). The *error* of a single reading determined from twenty identical plasma samples was found to be $\pm 4.7\ \%$ ($\pm\sigma$).

The percentage of K that has left the red cells at different times of storage is the simplest way of expressing the cell K loss. This method, however, was not considered satisfactory, because different blood samples had different hematocrit values (cord blood higher than adult blood) and because the concentration gradient for K changes during the storage. *The rate of K loss* was therefore calculated in the following way, with due consideration to the above factors.

Every blood sample was regarded as a system of two compartments separated by a membrane, the plasma being the one compartment (1) and the red cells the other (2). The red cell membrane was regarded as the membrane of the system. If the K concentration of compartment (1) is analysed, the following equations are used.

$$\frac{dn}{dt} = D\frac{A}{\delta}(C_2 - C_1) \tag{8}$$

$$dn = -V_2 \cdot dC_2 = V_1 \cdot dC_1 \tag{9}$$

$$V_1 C_1^0 + V_2 C_2^0 = V_1 C_1 + V_2 C_2 = (V_1 + V_2) C_m \tag{10}$$

where $D$ is the diffusion coefficient for K, $A$ the area across which the diffusion occurs, $\delta$ the thickness of the membrane, $V_1$ the volume of compartment (1), $V_2$ the volume of compartment (2), $C_1^0$ the K concentration in compartment (1) and $C_2^0$ the K concentration in compartment (2) at zero time, $C_1$ and $C_2$ respectively the K concentrations at the time $t$, $C_m$ is the average K concentration of the total system, and $n$ the amount of K entering the plasma.

By combining the equations (8), (9) and (10) we obtain

$$\frac{dC_1}{dt} = D\frac{A}{\delta}\left[\frac{V_1C_1^0 + V_2C_2^0 - C_1(V_1+V_2)}{V_1V_2}\right]. \qquad (11)$$

$DA/\delta$ can be substituted by a constant $k$, which is called the *K loss coefficient* and has the dimension volume time$^{-1}$. The $k$ in this two-compartment system is a measure of the K transport across the membrane. After integrating equation (11) and solving for $k$, we obtain

$$k = \frac{V_1V_2}{(V_1+V_2)t}\ln\frac{V_2(C_2^0 - C_1^0)}{(V_1+V_2)(C_m - C_1)}. \qquad (12)$$

In this equation all parameters can be determined.

## Results

### *Storage at +4°C*

With the criterion used in this investigation the heparinized fetal red cells seem to disintegrate faster than adult red cells when stored in their own plasma at +4° C.

The fetal red cells showed a more rapid *spontaneous hemolysis* than the adult red cells (Table 7).

TABLE 7

The percentage of spontaneous hemolysis after different times of storage at +4°C.

| | No. of cases | 0–24 hours | No. of cases | 0–120 hours | No. of cases | 0–264 hours |
|---|---|---|---|---|---|---|
| Cord blood . . . . . | 10 | 0.5 % | 8 | 6.0 % | 3 | 16.0 % |
| Adults . . . . . | 9 | 0 % | 7 | 0.6 % | 6 | 6.6 % |

The decrease in *mechanical resistance* occurred at a faster rate in cord blood than in adult blood (Table 8).

TABLE 8

The mean difference in mechanical resistance after different times of storage at +4°C.

| | No. of cases | 0–24 hours | No. of cases | 0–120 hours | No. of cases | 0–264 hours |
|---|---|---|---|---|---|---|
| Cord blood . . . . . | 6 | −4.8 % | 5 | −8.6 % | 4 | −17.5 % |
| Adults . . . . . | 9 | −1.1 % | 5 | −3.2 % | 5 | −15.0 % |

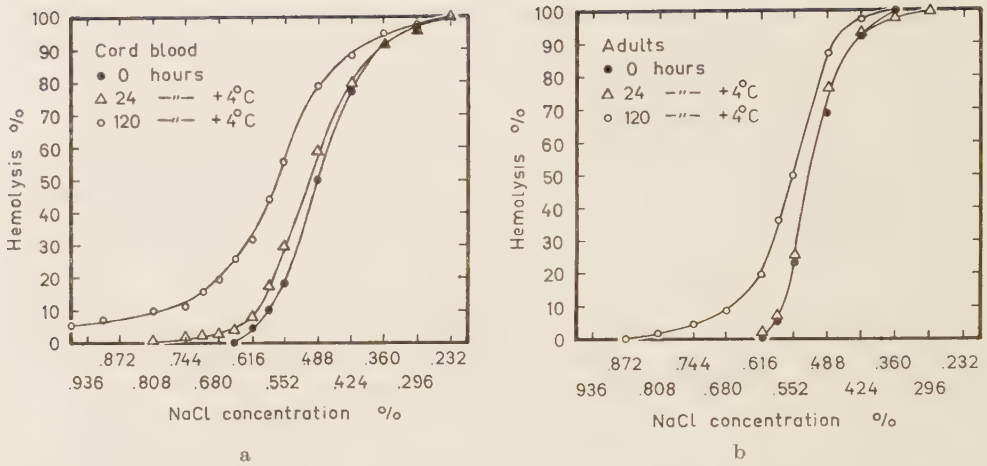



Fig. 29. Mean percentage hemolysis of 10 cord blood (fig. 29 a) and 9 adult blood samples (fig. 29 b) at different NaCl concentrations before and after different times of storage at + 4° C.

There was also a faster decrease in *osmotic resistance* of the fetal red cells than of the adult cells (Fig. 29). After a storage of 24 hours the osmotic resistance of 10 cord blood samples showed a greater decrease than the osmotic resistance of 9 adult blood samples. The osmotic resistance curves did not show a parallel shift to the left during cold storage. Some cells showed a more rapid decrease in osmotic resistance. Whether the cells that were more sensitive to storage were the same as those that were less resistant from the beginning is not possible to say. If the shift is expressed as the mean area between the osmotic resistance curves at zero time and at 24 hours, a "shift area" of 4.3 $cm^2$ was found for the cord blood, while the adult "shift area" was 0.7 $cm^2$. The difference between these values was significant ($P < 0.001$). The corresponding values for a storage of 120 hours were 16.3 $cm^2$ and 9 $cm^2$ ($P < 0.05$). Because it was difficult to obtain sufficient amounts of cord blood, only 3 cord blood samples were investigated after a storage of 264 hours. No definite comparison with the adult blood was thus possible. The general tendency, however, seemed to be the same also after this time of storage.

A comparison of *microphotographs* of fetal and adult red cells at the start and at the end of 264 hours cold storage showed that the number of crenated and distorted red cells was greater in the cord blood samples than in the adult samples (Figs. 30 and 31). The same difference was also observed between fetal and adult red cells stored for 120 hours at + 4° C.

During cold storage a decrease in the *red cell diameter* also occurred (Fig. 32). No remarkable difference between fetal and adult red cells was observed.

The *loss of potassium* from fetal red cells occurred at a faster rate than from

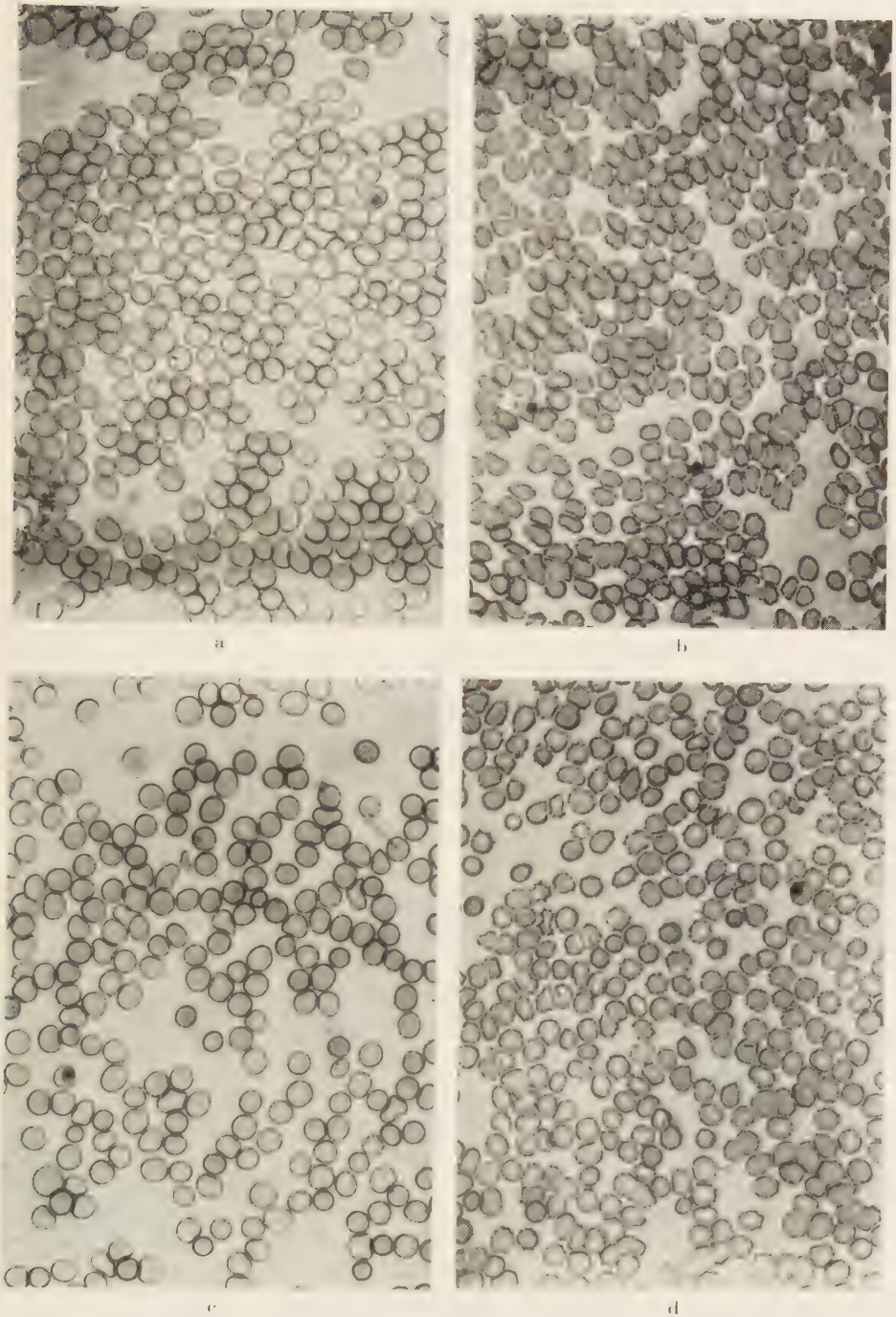
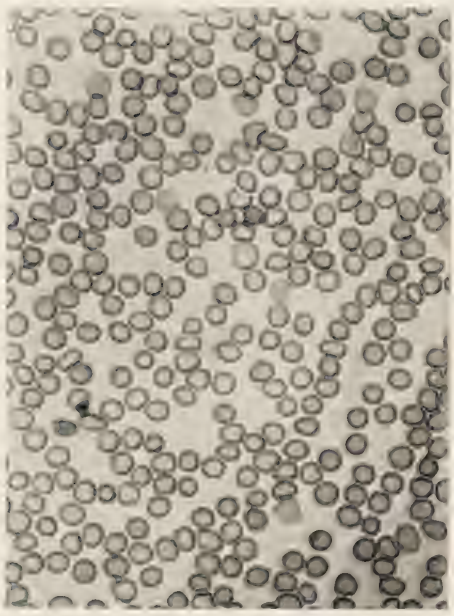
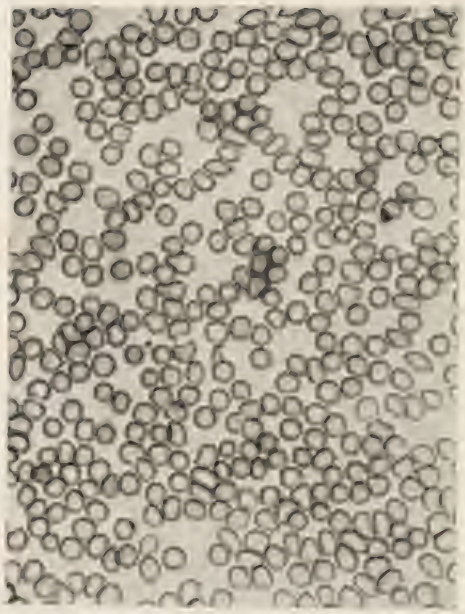


Fig. 30. Microphotographs of the red cells of two cord blood samples before (fig. 30 a and c) and after (fig. 30 b and d) 264 hours storage at +4 °C.



a



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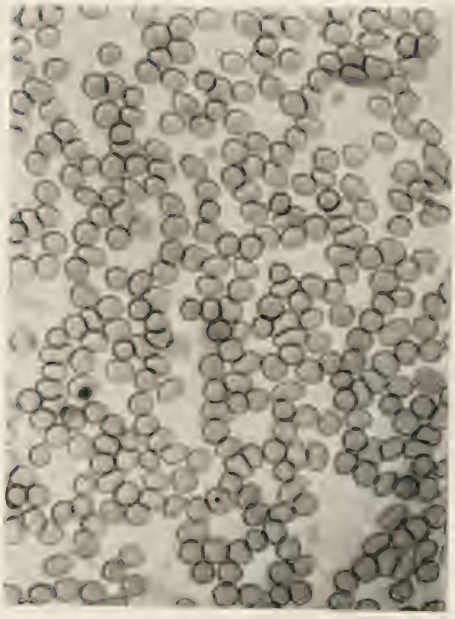
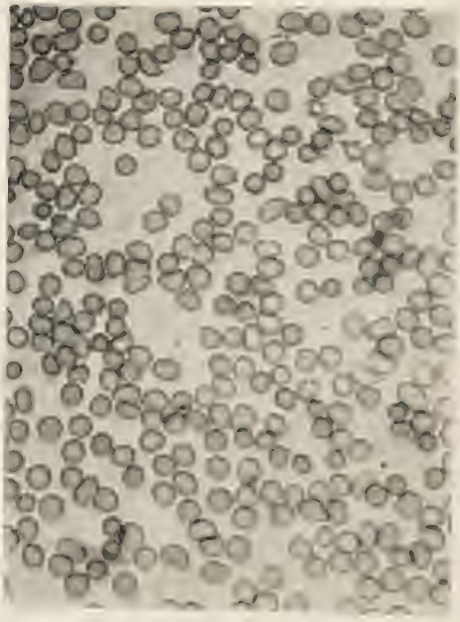


c



d

Fig. 31. Microphotographs of the red cells of two adult blood samples before (fig. 31 a and c) and after (fig. 31 b and d) 264 hours storage at +4 °C.

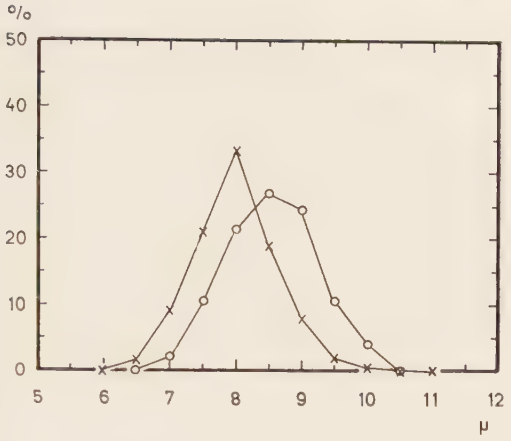



Fig. 32. The red cell diameter distribution of a cord blood sample before (O) and after (×) 264 hours storage at +4 C.

adult red cells during storage at +4° C. In Fig. 33 the rate of K loss from red cells of 3 cord blood and 3 adult blood samples is given as percentage of the initial total K content. The fetal red cells lost their K more rapidly than the adult cells. The difference seemed more pronounced during the first 24 hours. A closer study of this part of the curve was therefore considered to be of a special interest.

The result of such a study is shown in Fig. 34. During this initial phase of storage the rate of K loss of the fetal red cells was great in comparison with the adult red cells. Because of reasons mentioned above (p. 68) these curves, although they give a true picture of the K loss in the individual cases, are not appropriate for a comparison of the K loss rate of different red cell samples. Therefore the K loss coefficient, $k$, was calculated according to equation (12). For 10 cord blood samples a mean $k$ of 0.325 was found, while the $k$ of 10

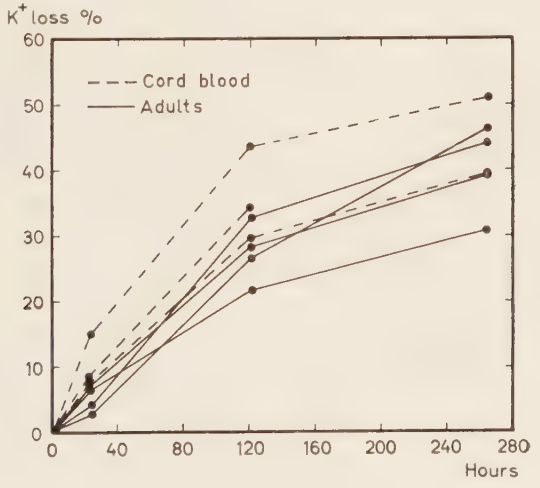
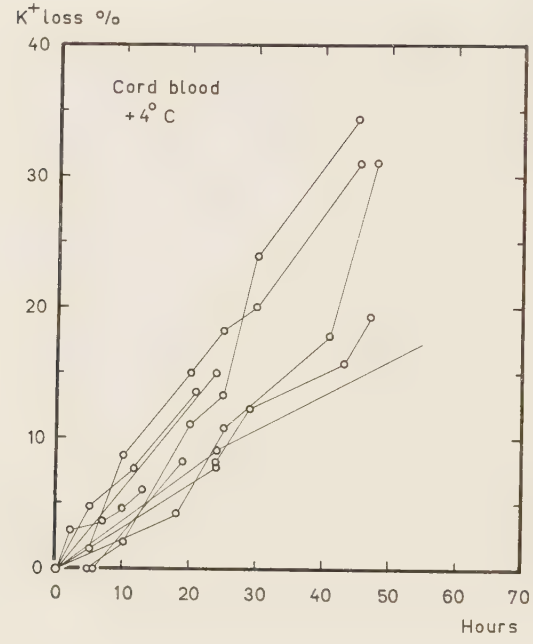
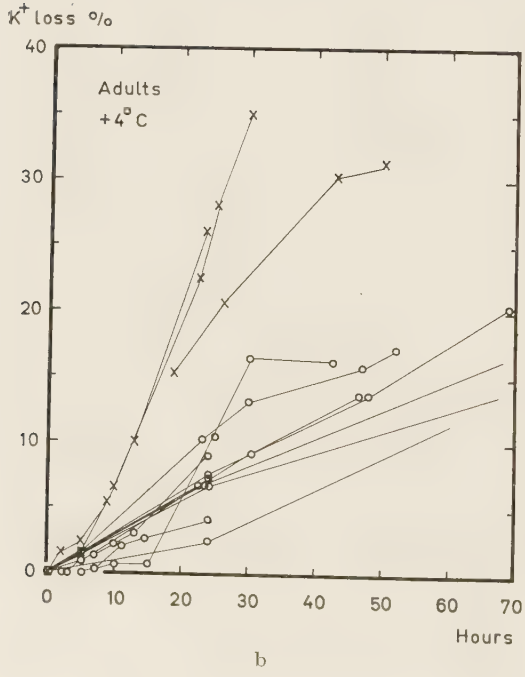



Fig. 33. The rate of K loss from red cells of cord blood and adult blood during storage at +4 °C.




a




b

Fig. 34. The rate of K loss from red cells of cord blood (fig. 34 a) and adult blood (fig. 34 b) during storage at +4 °C. The curves of two "abnormal" adults are indicated by ×.

TABLE 9

The "K loss coefficient" of cord blood and adult blood at $+4^{\circ}$C.

| Cord whole blood | | | Adult whole blood | | |
|---|---|---|---|---|---|
| Case | Hours of storage | $k$ | Case | Hours of storage | $k$ |
| 1. | 5 | 0.649 | 1. | 25 | 0.234 |
| | 11.5 | 0.483 | | 30 | 0.195 |
| | 21 | 0.508 | | 42.5 | 0.243 |
| 2. | 10 | 0.235 | 2. | 23 | 0.193 |
| | 13 | 0.247 | | 31 | 0.158 |
| 3. | 10 | 0.092 | | 47 | 0.162 |
| | 20 | 0.279 | 3. | 23 | 0.206 |
| | 30 | 0.453 | | 30 | 0.197 |
| | 45 | 0.399 | | 47 | 0.150 |
| 4. | 10 | 0.317 | | 52 | 0.159 |
| | 20 | 0.427 | 3. | 24 | 0.146 |
| | 25 | 0.406 | | 48 | 0.139 |
| | 30 | 0.369 | | 72 | 0.159 |
| | 45.5 | 0.454 | 4. | 24 | 0.096 |
| 5. | 19 | 0.173 | | 120 | 0.274 |
| | 24 | 0.164 | | 264 | 0.381 |
| | 43 | 0.313 | 4. | 14.5 | 0.089 |
| | 47 | 0.234 | 5. | 24 | 0.142 |
| 6. | 18 | 0.031 | | 120 | 0.118 |
| | 25 | 0.187 | 6. | 24 | 0.029 |
| | 41 | 0.198 | | 120 | 0.052 |
| | 48 | 0.532 | 7. | 24 | 0.162 |
| 7. | 24 | 0.286 | | 120 | 0.183 |
| | 120 | 0.388 | 8. | 17 | 0.113 |
| 8. | 24 | 0.485 | | 24 | 0.123 |
| 9. | 24 | 0.284 | | 48 | 0.131 |
| | 120 | 0.394 | 9. | 17.5 | 0.205 |
| 10. | 22 | 0.227 | | 23 | 0.243 |
| | 45.5 | 0.259 | | 48 | 0.139 |
| | 71 | 0.269 | | 96 | 0.134 |
| | | | 10. | 10 | 0.136 |
| | | | | 24 | 0.118 |
| | | | | 48 | 0.108 |
| Mean $k = 0.325 \pm 0.026$ | | | Mean $k = 0.161 \pm 0.012$ | | |

adult blood samples was 0.161 (Table 9). The difference was significant ($P < 0.001$). In two other healthy adults the rate of the K loss was very much increased compared with the other adults and even with cord blood (Fig. 34 b). The mean $k$ for these two adults was 0.432. The difference was so great

TABLE 10

The "K loss coefficient" of fetal and adult red cells suspended in Salidex$^{R}$ at $+4^{\circ}$C.

| Cord blood red cells + Salidex$^{R}$ | | | Adult red cells + Salidex$^{R}$ | | |
|---|---|---|---|---|---|
| Case | Hours of storage | $k$ | Case | Hours of storage | $k$ |
| 10. | 22 | 0.207 | 2. | 22.5 | 0.105 |
| | 45 | 0.253 | | 28 | 0.146 |
| | 71 | 0.251 | | 53 | 0.150 |
| 11. | 8 | 0.266 | | 101 | 0.100 |
| | 11 | 0.251 | 3. | 8 | 0.168 |
| 12. | 17 | 0.241 | | 11 | 0.191 |
| | 24 | 0.260 | 4. | 11 | 0.119 |
| | 48 | 0.349 | | 13.5 | 0.132 |
| 13. | 19 | 0.226 | 9. | 17,5 | 0.093 |
| | 27 | 0.353 | | 23 | 0.146 |
| | 48 | 0.374 | | 48 | 0.151 |
| 14. | 19 | 0.226 | | 96 | 0.137 |
| | 27 | 0.353 | 11. | 18.5 | 0.253 |
| | 48 | 0.374 | | 26 | 0.242 |
| | | | | 48 | 0.158 |
| Mean $k = 0.285 \pm 0.018$ | | | Mean $k = 0.153 \pm 0.012$ | | |

($k = 0.161 \pm 0.012$ and 0.432 respectively) that it seemed permissible to regard the K loss of these two adults as abnormal. A more detailed study of these red cells would be of interest, but has been considered outside the scope of this investigation.

The *K loss of washed fetal and adult red cells* suspended in Salidex$^{R}$ showed the same difference as the red cells of whole blood. The mean K loss coefficient, $k$, of fetal red cells was 0.285, while $k$ of adult red cells was 0.153 (Table 10). The difference between these values is significant ($P < 0.001$).

The K loss coefficient, $k$, of the *red cells of 4 newborn infants aged 23 to 60 hours* was 0.390, 0.462, 0.534, and 0.446, which indicates a more rapid K loss during the postnatal period than at birth.

*Storage at $+37^{\circ}C$*

Also when stored at $+37^{\circ}$ C characteristic differences were observed between fetal and adult red cells.

No *spontaneous hemolysis* was observed in 7 adult blood samples stored for 24 hours at $+37^{\circ}$ C, while 4 of 7 cord blood samples showed a spontaneous hemolysis between 1 and 11 %.

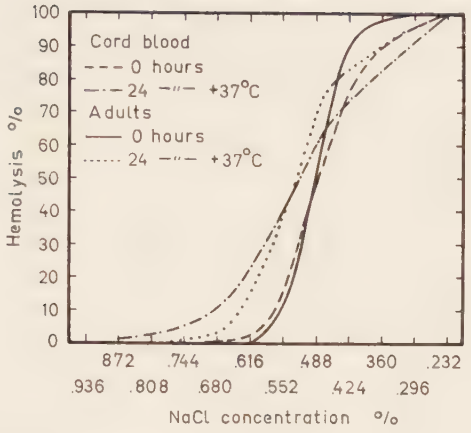


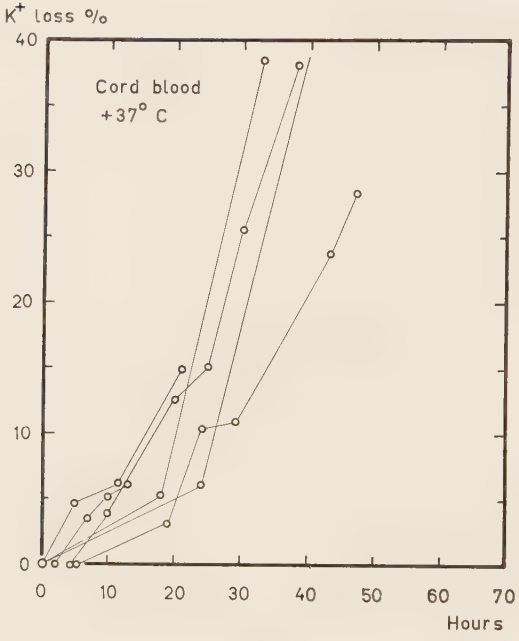

Fig. 35. Mean percentage hemolysis of 7 cord blood and 7 adult blood samples at different NaCl concentrations before and after 24 hours storage at +37 °C.

During a 24 hours storage at +37° C the change in *osmotic resistance* was different from that observed during storage at +4° C. The majority of the red cells showed a decreased osmotic resistance in the same way as at +4° C, but a minority of cells showed an *increased* osmotic resistance after the storage (Fig. 35). The *mean decrease* in the osmotic resistance of 7 cord blood samples was greater than that of 7 adult samples, but the difference was not significant when expressed as the difference between the "shift areas" (10.1 $cm^2$ and 7.1 $cm^2$ respectively, $0.10 < P < 0.50$). The *mean increase* in osmotic resistance of the cord blood samples was not significantly greater than the resistance increase of the adult blood samples (3.0 $cm^2$ and 2.2 $cm^2$ respectively, $0.50 < P$).

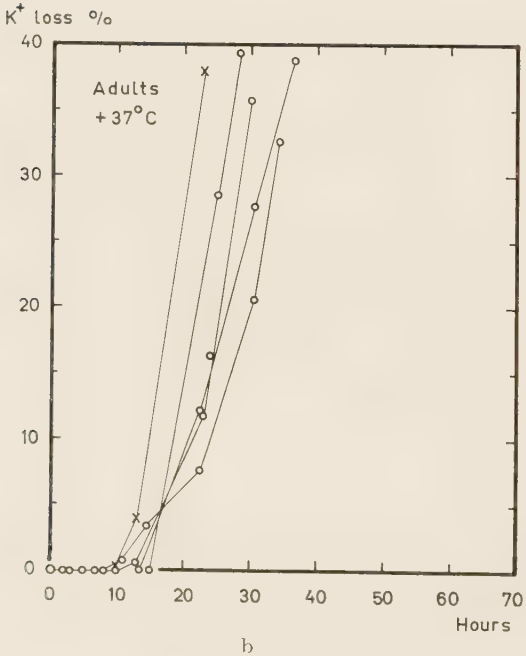
The *rate of K loss* of red cells stored for about 48 hours at +37° C is illustrated in Fig. 36. It is evident that the cord blood cells started to lose K earlier than the adult cells. Once the K loss of the adult red cells had started, the loss seemed to be more rapid than the K loss of cord blood cells. A mean K loss coefficient, $k$, of 0.362 was found for 6 cord blood samples (5 of these cases are represented in Fig. 36). In 4 adult blood samples the mean value was 0.613. Table 11 illustrates that $k$ is not constant during the storage but increases with the time. The red cells of one of the "abnormal" adults also showed an increased K loss rate at +37° C (Fig. 36 b).

*Storage at +4°C followed by a storage at +37°C*

When red cells were at first stored at +4° C for 24 hours and then at +37° C for 24 hours the osmotic resistance was changed as shown in Fig. 37. After the decrease during the cold storage there was a remarkable *increase* in osmotic resistance after the storage at +37°C. The effect was obtained both with fetal and adult red cells. No definite difference was found between the two




a




b

Fig. 36. The rate of K loss from red cells of cord blood (fig. 36 a) and adult blood (fig. 36 b) during storage at +37 °C. The curve of one "abnormal" adult is indicated by ×.

types of cells. The K loss of the cells was increased during the storage at 37° C when compared with the K loss during a continued storage at - 4° C and was about the same as during an immediate storage at + 37° C.

TABLE 11

The "K loss coefficient" of cord blood and adult blood at + 37°C.

| Cord whole blood | | | Adult whole blood | | |
|---|---|---|---|---|---|
| Case | Hours of storage | *k* | Case | Hours of storage | *k* |
| 1. | 11.5 | 0.394 | 1. | 25 | 0.750 |
| | 21 | 0.586 | | 30 | 1.537 |
| 2. | 10 | 0.114 | 2. | 23 | 0.330 |
| | 13 | 0.110 | | 31 | 0.633 |
| 4. | 10 | 0.202 | 3. | 23 | 0.241 |
| | 20 | 0.371 | | 30 | 0.712 |
| | 25 | 0.380 | | 47 | 0.890 |
| | 30 | 0.674 | 3. | 24 | 0.307 |
| 5. | 19 | 0.158 | 4. | 23 | 0.255 |
| | 24 | 0.183 | | 31 | 0.479 |
| | 29 | 0.201 | 4. | 14.5 | 0.147 |
| | 43 | 0.702 | | | |
| | 47 | 0.832 | | | |
| 6. | 18 | 0.170 | | | |
| 11. | 24 | 0.064 | | | |

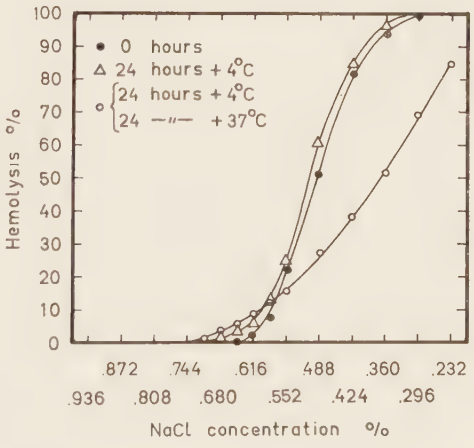



Fig. 37. Degree of hemolysis of a cord blood sample at different NaCl concentrations before storage, after 24 hours storage at + 4 °C and after 24 hours storage at + 4 °C followed by 24 hours storage at + 37 °C.

## Discussion

*Storage at $+4°C$*

Because the metabolic activity of the red cells is minimized during the cold storage and because whole blood and washed red cells show the same storage behaviour, the different storage behaviour of cord blood and adult blood must be attributed to differences between fetal and adult red cells, probably to membrane differences.

During cold storage of red cells the cell membrane becomes cation permeable, and Na and K move according to their concentration gradients. Because the rate of change of Na is greater than that of K, the total red cell base will increase successively. The cell membrane is still impermeable to the red cell proteins, which will thus exert an inside colloid osmotic pressure. These two factors, the total base increase and the colloid osmotic pressure of the proteins, cause a water uptake resulting in a swelling of the cells during cold storage. The decreasing red cell diameter and the decreasing osmotic resistance during cold storage depend probably on this swelling of the red cells. The rate of K loss is an expression of the permeability of the red cell membrane to K. The decreasing osmotic resistance during storage is mainly an expression of the difference in the diffusion rate of K and Na.

In the present investigation it was shown that the cation permeability of the fetal red cells is higher than that of adult red cells (Fig. 34, Table 9). It was also shown that the osmotic resistance decreases more rapidly in the fetal red cells than in the adult ones (Fig. 29). Because the fetal and adult red cells have the same cation concentration gradients and because the difference of diffusion area between fetal and adult red cells is small (cf. p. 56), it seems permissible to conclude that *these differences in cold storage behaviour of fetal and adult red cells depend on differences in the properties of the red cell membrane of these two types of cells.*

The mechanical resistance of cold stored fetal red cells decreases more rapidly than that of adult red cells stored in the same way (Table 8). Although the significance of the mechanical resistance test is not known, it seems probable that the difference between cold stored fetal and adult red cells is connected with membrane differences of the two types of cells, the membrane of fetal red cells becoming more vulnerable to mechanical trauma during storage. According to Shen, Castle and Fleming (1944) the degree of spheroidicity will influence the mechanical resistance. The higher degree of spheroidicity of the stored fetal red cells may thus also be regarded as a direct or indirect explanation.

*On the whole cord blood red cells seem to be more vulnerable to cold storage than adult red cells.* Also the microphotographs support this opinion (Figs. 30

and 31). The relatively high K loss during the postnatal period may be an indication of a greater number of vulnerable red cells during this time than at birth.

*Storage at + 37° C*

The delayed start of the K loss of adult red cells is the most remarkable difference found between fetal and adult red cells stored at + 37° C (Fig. 36). In cord blood the K loss starts during the first 5 hours, while in adult blood a K loss is at first observed after 10 to 15 hours storage. This implies that the fetal red cells lose their capacity of maintaining a concentration gradient for the K ions earlier than the adult red cells. The most likely explanation of this phenomenon is *an earlier failure of the metabolic activity of the cord blood red cells.*

When the metabolism fails the cation gradients can not be maintained. The cation equilibration proceeds faster at + 37° C than at + 4° C probably depending on the temperature difference. Judging from the osmotic resistance tests (Fig. 35) and from the degree of spontaneous hemolysis this process seems to be more rapid in fetal red cells than in adult red cells, even if the difference is not significant. It is, however, impossible to compare in detail the rates of the cation equilibration of fetal and adult red cells, because their metabolism fails after different times. The increasing K loss coefficient during the experiments is manifest evidence thereof (Table 11). No definite difference in the rate of cation equilibration of fetal and adult red cells stored at + 37° C can thus be proved.

It must also be remembered that other factors than the red cell properties may affect the red cells during the storage at + 37° C. Minimal bacterial contamination, evaporation of water, pH changes and different plasma constituents may affect the rate of deterioration. It is thus to be expected that the experimental conditions in themselves cause considerable variation in the results.

An interesting observation concerning red cells stored at + 37° C is that *the osmotic resistance of some of the red cells increases* (Fig. 35), while the resistance of the majority decreases. A similar increase in osmotic resistance, though affecting a greater number of cells, was observed on the occasion when red cells were at first stored at + 4° C and then at + 37° C (Fig. 37). The effect was the same both on fetal and on adult red cells. The real nature of this phenomenon has not been revealed. Because the K loss proceeds at the usual rate, it is not possible to look for the explanation of the increased osmotic resistance in a shrinking of the cells. It seems more likely that the hemoglobin of these red cells changes its state during these storage conditions and becomes osmotically inactive because of gelation or crystallization.

## Final Comment

The cord blood red cells differ markedly from the adult red cells with respect to the physical properties studied above. There are definite indications of differences in the red cell membrane of the two cell types. Some of the cord blood red cells, at least, seem also to be more susceptible to different in vitro stresses (mechanical trauma, cold storage and hypotonicity). Whether this greater vulnerability of the cord blood red cells in vitro also implies a shorter in vivo survival cannot be proved by the present investigation. Transfusion experiments with cord blood (MOLLISON, 1951) support the view that some fetal red cells have a shorter life time in vivo than normal adult red cells. Accordingly a certain correspondence can be said to exist between the in vitro resistance of red cells and their in vivo survival. Experiments on red cells from cases with different hemolytic disorders, not treated in this paper, point in the same direction. It is, however, quite possible that this apparent parallelism is nothing but a coincidence. The investigations of GABRIO and FINCH (1954), though covering only the first 24 post-transfusion hours, seem to support such an interpretation. Because they also found that young red cells were more suspectible to cold storage than older cells, it can be questioned whether the comparatively great number of young red cells in cord blood explains the higher susceptibility of cord blood to cold storage. It should, however, be emphazised that the present investigation deals with untreated whole blood, while most of the other experiments are performed with whole blood suspended in acid-citrate-dextrose solution, in which the rate of deterioration is much more slow.

## Summary

The main object of the investigation was to study some physical properties of the fetal red cells in order to find out whether, or to what extent, fetal red cells differed from adult red cells.

A short survey of the properties of adult and fetal red cells with special reference to the characteristic shape and hemoglobin differences was first given (Chapter I).

The *osmotic resistance* was studied in buffered, hypotonic NaCl solutions (pH 6.8; temp. $+4°$ C), and the amount of liberated hemoglobin was used

as a measure of the degree of hemolysis. Percentage hemolysis curves were thus obtained. A characteristic variation of the osmotic resistance at different ages was observed. The majority of the red cells of young fetuses showed a lower resistance than the red cells of cord blood of full-term infants. A continuous increase in resistance towards the cord blood values was noticed during the fourth and fifth months of gestation. The mean osmotic resistance of cord blood and adult blood did not differ. The span of hemolysis, however, was greater in cord blood, the resistant cells especially being more numerous than in adult blood. During the whole childhood the resistant cells were more numerous than in adult blood. The greatest number of resistant cells was found during the first days of life and at the end of the first year of life. During the first six months of life the resistance distribution was skew, thereafter it was practically normal. At about puberty a definite shift to the adult red cell type seemed to occur.

Special attention was paid to the methodological problems, to the statistical treatment of percentage hemolysis curves, and to the significance of the osmotic resistance test. The changes in osmotic resistance were interpreted as depending, at least partly, on variations in the red cell shape. It was found impossible to draw any definite conclusions from the osmotic resistance as to the age or to the in vivo survival of different normal red cells. The oldest cells, however, seemed to belong to the osmotically most resistant part of the red cell population. The changes in osmotic resistance were regarded as indications of variations in the composition of the red cell population or in the red cell environment (Chapters II and III).

The rate of volume increase of red cells in buffered, hypotonic NaCl solutions (pH 7.0; temp. $+20°$ C) was measured photometrically and was regarded as a measure of the *rate of entrance of water* into the red cells. The *permeability coefficient* of water was calculated from these experiments. A greater value was found for adult red cells than for cord blood red cells, $22.1 \times 10^{-3}$ cm sec.$^{-1}$ and $11.7 \times 10^{-3}$ cm sec.$^{-1}$ respectively. The difference was interpreted as depending on differences in the membrane of the two types of cells. The obtained permeability coefficient of adult red cells was discussed in relation to the present conception of the red cell membrane structure. The information obtained by the permeability studies was found to be in good accordance with the opinion derived from earlier chemical and electron microscope investigations. Because different methods gave varying permeability coefficients for the same blood, methodological problems concerning the determination of the permeability coefficient were also treated (Chapter IV).

When a blood sample is subjected to a standardized *mechanical trauma*, some red cells will burst and hemolyse. The percentage of undestroyed red

cells was regarded as a measure of the mechanical resistance. A decreased mechanical resistance was found at birth and during the first two to three months of life. This greater vulnerability may indicate a shorter in vivo survival of the cord blood red cells, but there is no proof of such a presumption. With regard to normal red cells the mechanically most resistant cells also seemed to be the osmotically most resistant ones (Chapter V).

During *cold storage* (+ 4° C) of whole blood the glycolytic metabolism of the red cells is minimized. As a result of the failing metabolism the red cells swell, their osmotic resistance decreases and they lose potassium and gain sodium in accordance with their diffusion coefficients and concentration gradients. Judging from changes in the osmotic resistance and in the cation permeability, the cord blood red cells equilibrated their cations faster than the adult red cells. The difference was attributed to membrane differences between the two types of cells (Chapter VI).

During *storage at + 37° C* glycolysis at first proceeds at the in vivo rate, and the normal in vivo concentration gradients of the cations are maintained. Judging from the potassium loss curves during storage at + 37° C the metabolism of the cord blood red cells seemed to fail earlier than that of the adult red cells. After the metabolic failure it was not possible to prove any difference in the rate of cation equilibration between cord blood and adult red cells (Chapter VI).

## Zusammenfassung

In der vorliegenden Arbeit werden Erythrocyten aus fetalem, menschlichem Blut mit solchen aus Erwachsenenblut bezüglich ihrer physikalischen Eigenschaften verglichen.

Unterschiede der Form und des Hämoglobingehalts werden eingangs beschrieben (Kapitel I).

Zur Untersuchung der *osmotischen Resistenz* dienten hypotonische NaCl-Lösungen von abgestufter Ionenstärke (pH 6.8, gepuffert; Temp. + 4° C). Der Hämolysegrad wurde anhand der Menge des freien Hämoglobins ermittelt. Es ergaben sich auf diese Weise %-Hämolysekurven. Die osmotische Resistenz der Erythrocyten war in charakteristischer Weise vom Alter der untersuchten Individuen abhängig. So zeigten rote Blutkörperchen von jungen Feten mehrheitlich eine geringere Resistenz als solche aus dem Nabelschnurblut von ausgetragenen Kindern. Der Haupt-Anstieg der Resistenz fiel in den 4.–5. Schwangerschaftsmonat. Nabelschnur-Erythrocyten zeigten den gleichen Mittelwert der osmotischen Resistenz wie Erwachsenen-Erythrocyten, hingegen bestanden bei den ersteren grössere Abweichungen vom Mittelwert. Während der ganzen Dauer des Kindesalters lag die Resistenz

über dem Normalwert für Erwachsene, wobei Höchstwerte in den ersten Lebenstagen und am Ende des ersten Lebensjahres zu verzeichnen waren. Im Laufe der ersten 6 Lebensmonate war die Resistenzverteilung nicht glockenförmig, nachher jedoch normal. Zur Zeit der Pubertät war ein Wechsel zum Erythrocytentyp Erwachsener nachzuweisen.

Probleme der experimentellen Methodik werden hier eingehend besprochen; es wird auf die statistische Behandlung der Hämolysekurven eingegangen, und die Bedeutung des osmotischen Resistenztests wird diskutiert. In bestimmten Fällen ist es möglich, Veränderungen der Erythrocytenform für beobachtete Resistenzveränderungen verantwortlich zu machen. Auch scheinen die ältesten Zellen einer Population zu den osmotisch resistentesten zu gehören. Im allgemeinen darf aber aus dem Wert der osmotischen Resistenz nicht auf das Alter der Erythrocyten geschlossen werden, noch dürfen die Aussichten für ein Überleben in vivo abgeschätzt werden. Als massgebend für eine Änderung der osmotischen Resistenz kommen in Frage eine veränderte Zusammensetzung der Erythrocyten-Population oder eine veränderte Zusammensetzung des Erythrocyten-Milieu. (Kapitel II und III).

Die Geschwindigkeit, mit der das Erythrocytenvolumen in hypotonischer NaCl-Lösung zunahm, wurde photometrisch gemessen (pH 7, gepuffert; Temp. 20° C). Daraus liess sich der *Permeabilitätskoeffizient für Wasser* ermitteln. Dieser war bei Erythrocyten von Erwachsenen grösser als bei solchen aus Nabelschnurblut ($22{,}1 \times 10^{-3}$ cm $sec^{-1}$ gegenüber $11{,}7 \times 10^{-3}$ cm $sec^{-1}$), woraus auf unterschiedliche Membraneigenschaften dieser beiden Zelltypen zu schliessen ist. Die hier ermittelte Durchlässigkeit des Erwachsenen-Erytrocyten für Wasser steht im Einklang mit gewissen Ansichten über die Membranstruktur, wie sie aus früheren chemischen und elektronenmikroskopische Untersuchungen herzuleiten sind. Da verschiedene Methoden verschiedene Permeabilitätskoeffizienten für Wasser ergaben, werden hier methodische Probleme ebenfalls auseinandergesetzt.

Wenn ein *mechanisches Trauma* von geeignet gewählter Intensität auf eine Blutprobe einwirkt, platzen einige Blutkörperchen und hämolysieren. Die Prozentzahl der unversehrten Erythrocyten ist dann ein Mass für die mechanische Resistenz. Letztere erwies sich zur Zeit der Geburt und in den 2–3 folgenden Monaten als relativ niedrig. Dieser Befund mag mit der Tatsache in Beziehung stehen, dass einige Nabelschnur-Erythrocyten eine relativ geringe in vivo Überlebenszeit aufweisen. In einer Population von „normalen" Erythrocyten scheinen die mechanisch widerstandsfähigsten Zellen zugleich die osmotisch resistentesten zu sein (Kapitel V).

*Das Aufbewahren von Gesamtblut in der Kälte* ( + 4° C) ist mit einer starken Herabsetzung des glykolytischen Stoffwechsels verbunden; die roten Blutkörperchen schwellen dabei an; ihre osmotische Resistenz nimmt ab; sie

verlieren Kalium und nehmen Natrium auf. Die Geschwindigkeit des Ionenaustausches hängt von den Konzentrationsunterschieden sowie von den Diffusionskoeffizienten ab. Aus Messungen der osmotischen Resistenz und aus Kationenbestimmungen ist zu schliessen, dass bei Nabelschnur-Erythrozyten die Einstellung eines neuen Ionengleichgewichts rascher vor sich geht als bei Erwachsenen-Erythrozyten. Die Membranen dieser beiden Zelltypen scheinen sich also auch bezüglich der Ionenpermeabilität zu unterscheiden (Kapitel VI).

*Das Aufbewahren von Blut bei 37° C* ist anfänglich nicht mit einer Abnahme des in vivo Wertes der Glycolyse verbunden, und die normalen Kationen-Gradienten blieben erhalten. Die intracelluläre Kalium-Konzentration begann im Nabelschnurblut bereits nach kürzerer Zeitdauer abzusinken als im Erwachsenenblut; dies deutet darauf hin, dass im Nabelschnurblut der „normale" Stoffwechsel rascher zusammenbricht. Nach völligem Versagen des Stoffwechsels konnte an den genannten Zelltypen kein Unterschied in der Geschwindigkeit des Ionenaustausches mehr festgestellt werden (Kapitel VI).

## Résumé

Le principal objet de cette étude a été d'examiner quelques propriété physiques des globules rouges fœtaux afin de découvrir si les globules rouges fœtaux différaient des globules adultes et dans quelle mesure.

Un court examen des propriétés des globules rouges adultes et fœtaux, en se référant particulièrement à la forme caractéristique et aux différences d'hémoglobine, a d'abord été fait (Chapitre I).

*La résistance osmotique* a été étudiée dans des solutions tamponnées, hypotoniques de ClNa (pH 6,8; Temp. + 4°C), et la quantité d'hémoglobine libérée a été utilisée comme mesure du degré d'hémolyse. Des courbes du pourcentage d'hémolyse ont ainsi été obtenues. La majorité des globules rouges des jeunes fœtus ont montré une résistance plus faible que ceux du sang du cordon ombilical des enfants à terme. Un accroissement continu de résistance vers les valeurs du cordon a été enregistré durant le 4è et le 5è mois de la gestation. La résistance moyenne du sang du cordon et celle du sang adulte ne diffèrent pas. La «span» d'hémolyse, cependant, était plus grand dans le sang du cordon, les cellules résistantes étant, en particulier, plus nombreuses que dans le sang adulte. Pendant toute l'enfance les cellules résistantes se sont montrées plus nombreuses que dans le sang adulte. Le plus grand nombre de cellules résistantes a été trouvé pendant les premiers jours de la vie et à la fin de la première année de vie. Pendant les six premiers mois de vie, la courbe de distribution des résistances était assymétrique, en-

suite elle était pratiquement normale. Aux environs de la puberté, une transformation définitive en globules rouges adultes semble survenir.

Une attention spéciale a été portée aux problèmes méthodologiques, au traitement statistique des courbes de pourcentage d'hémolyse et à la signification du test de résistance osmotique. Les changements dans la résistance osmotique ont été interprétés comme dépendant, au moins en partie, de variations dans la forme des globules rouges. Il a été impossible de tirer des conclusions définitives de la résistance osmotique en ce qui concerne l'âge ou la survie in vivo de différents globules rouges normaux. Les plus vieilles cellules, cependant, semblaient appartenir, au point de vue osmotique, à la partie la plus résistante des globules rouges. Les changements dans la résistance osmotique ont été considérés comme des indications de variations dans la composition de la population cellulaire ou dans le milieu environnant (Chapitres II et III).

Le taux d'accroissement de volume dans les globules rouges en solution tamponnée et hypotonique de NaCl (pH 7,0; Temp. $+20°C$) a été mesuré photométriquement et considéré comme une mesure de la *vitesse de pénétration de l'eau. Le coefficient de perméabilité de l'eau* a été calculé d'après ces expériences. On a trouvé une valeur plus grande pour les globules rouges adultes que pour ceux du cordon, respectivement $22.1 \times 10^{-3}$ cm sec.$^{-1}$ et $11.7 \times 10^{-3}$ cm sec.$^{-1}$. La différence a été interprétée comme dépendant de différences dans la membrane des deux types de cellule. Le coefficient de perméabilité obtenu pour les globules rouges adultes a été discuté en rapport avec la présente conception de la structure de la membrane cellulaire. On a trouvé que les renseignements tirés des études de perméabilité s'accordent bien avec l'opinion qui découle de recherches effectuées antérieurement par voie chimique et au moyen du microscope électronique. Du fait que les différentes méthodes ont donné des coefficients de perméabilité différents pour le même sang, on a également étudié les problèmes méthodologiques concernant la détermination du coefficient de perméabilité (Chapitre IV).

Quand un échantillon de sang est soumis à un traumatisme mécanique standard, quelques globules rouges éclatent et s'hémolysent. Le pourcentage de globules rouges non détruits est une mesure de la résistance mécanique. On a observé une résistance mécanique plus faible à la naissance et durant les deux ou trois premiers mois de vie. On a suggéré que cette plus grande vulnérabilité était parallèle à une plus courte survie in vivo des globules rouges de cordon. Par comparaison avec les globules rouges normaux, les globules les plus résistants mécaniquement ont semblé être aussi les plus résistants du point de vue osmotique (Chapitre V).

Si l'on *conserve au froid* ($+4°$) du sang entier, le métabolisme glycolytique des globules rouges est diminué. Par suite d'un métabolisme moindre, les

globules rouges se gonflent, leur résistance osmotique décroît, ils perdent du potassium, et gagnent du sodium, conformément à leurs coefficients de diffusion et à leurs gradients de concentration. A en juger par les changements dans la résistance osmotique et dans la perméabilité aux cations, les globules rouges du cordon ont équilibré leurs cations plus vite que les globules rouges adultes. On a attribué les différences aux différences de membranes entre les deux cellules (Chapitre VI).

Si on *conserve les globules à +37°*, la glycolyse se produit d'abord a la même vitesse qu'in vivo, et les gradients de concentration des cations observés in vivo sont maintenus. A en juger par les courbes de perte de potassium pendant le sejou à +37°, le metabolisme des globules ronges de sang du cordon a semble diminuer plus tôt que celui des globules rouges normaux. Après la diminution du metabolisme, il n'a ete possible de mettre en évidence aucune différence dans la vitesse de mise en équilibre des cations entre le sang du cordon et les cellules adultes (Chapitre VI).

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